CHAPTER 2

MATERIALS AND METHODS
2.1. Silkworm Breeds and their Yield Potentials:

Four mulberry silkworm races viz., Pure Mysore, Nistari, NB₄D₂ & CSR₂ and two hybrid (♀ Pure Mysore x ♂ CSR₂ and ♀ Nistari x ♂ NB₄D₂) silkworms were selected as experimental system in the present investigation. Disease free layings of pure races were obtained from germplasm of the Department of Studies in Sericultural Sciences, University of Mysore, Mysore, were incubated at 24° ± 1° C temperature and 80-85% relative humidity. The silkworm rearing was conducted in the laboratory following four feeding schedule (Krishnaswami et al., 1973 and Krishnaswami, 1978 a, b, 1979). Fresh mulberry leaves of M-5 variety harvested from the adjoining garden, *ad libitum* were provided as food.

The experimental batches were allowed to complete larval development, spinning and pupation. Of the cocoons harvested, only uniform and healthy cocoons were selected. These cocoons were preserved race wise at a room temperature of 25± 2° C and relative humidity of 75 ± 5% (Tazima 1962; Jolly 1983; and Narasimhanna 1988). The emergence of moths started on 10th day. The healthy male and female moths of each batch were collected separately and allowed to mate for three hours. The moths were decoupled to prepare disease free eggs. The female moths of each treatment were allowed to lay eggs on the egg cards at room temperature of 25 ± 2° C and relative humidity of 75 ± 5%. Later, the egg cards were collected and surface sterilized with 2% formaldehyde solution for 5 minutes, washed in water and shade dried. The laying of CSR₂ and NB₄D₂ were treated with hydrochloric acid with specific gravity of 1.075 at a temperature of 46.1°C for 5 minutes as suggested by Tazima (1962) and Tanaka (1964). The eggs were incubated as per the procedure suggested by Krishnaswami (1979). The hybrid layings were prepared in the laboratory ((Tazima 1962; Jolly 1983; and Narasimhanna 1988). The pure races as well as hybrid silkworm batches were reared under the conditions described above during pre monsoon, monsoon and post
monsoon seasons of the year. Five hundred larvae from each breed were kept in triplicate after 3rd moult.

The economic parameters selected for present study included fecundity, weight of fifth instar larva, larval duration, single cocoon weight, single shell weight, shell ratio, filament length, denier and renditta.

The brief description of the economic traits and methods used as stated above are as follows:
a) **Fecundity:** This character represents the number of eggs laid by a female moth after mating.
b) **Weight of fifth instar larvae:** It represents the weight of twenty five randomly selected healthy and robust larvae weighed one day earlier to spinning.
c) **Larval Duration:** This trait represents the total duration in hours of rearing period of silkworm from the time of hatching to that of spinning.
d) **Single cocoon weight:** This represents the average weight of randomly selected twenty five cocoons in grams.
e) **Single shell weight:** This indicates the total quantity of silk in grams from twenty five cocoons selected randomly.
f) **Shell ratio:** It is the ratio between shell weight and cocoon weight. It is calculated and expressed in percentage from random samples of twenty five cocoons.

\[
\text{Shell percentage} = \frac{\text{Total weight of shell}}{\text{Total weight of cocoon}} \times 100
\]
g) **Filament length:** Total length of filament of single cocoon reeled using epprouvette (a reeling device for mono cocoon reeling). Mean values of such twenty five observations.
h) **Denier:** Denier is the thickness of the filament and was calculated using the following formula (Mahesha and Honnaiah, 1999).

\[
\text{Denier} = \frac{\text{Weight of the reeled silk}}{\text{Length of the reeled silk}} \times 9000
\]

i) **Renditta:** Renditta is the unit of fresh cocoons required to produce one unit of raw silk and was calculated using the following formula.

\[
\text{Renditta} = \frac{\text{Units of raw silk produced}}{\text{Units of fresh cocoons used}}
\]

Evaluation of these characters were carried out for both pure races as well as hybrid silkworms during three seasons viz., pre monsoon, monsoon and post monsoon seasons of the year. For all the experimental batches the procedure followed for evaluation was same as described earlier.

**2.2. Statistical Analysis**

The experimental data were statistically analyzed through SPSS by one way ANOVA (Fisher and Yates 1953) and Scheffe’s post hoc test (Scheffe, 1959) wherever they were applicable.
2.2. CHEMICALS

2.2.1. Substrates:

Substrates like starch, sodium succinate, paranitrophenol phosphate, paranitropheyl acetate, alpha napthyl acetate, beetanapthyl acetate were from Himedia, India.

2.2.2. Standard Proteins:

Molecular weight markers for SDS-PAGE were from Genie, India.

2.2.3 Reagent For Electrophoresis:

Ammonium persulphate (APS), acrylamide (electrophoresis grade), N-N’-methylene bis acrylamide, sodium dodesyl sulphate (SDS), N’,N’,-tetramethylethylenediamine (TEMED) and Tris [Tris (hydroxyl methyl) amino ethane] were from SRL India. Coomassie brilliant blue R-250 and beta mercaptoethanol were from Himedia, India.

2.2.4. Other Chemicals:

Unless otherwise stated all other chemicals used in the present study were of analytical grade obtained from either SRL or Merck or Qualigens or Himedia or Merck, India.
2.3. BIOCHEMICAL STUDIES

This part deals with the biochemical studies of selected silkworm breeds. The fifth instar larvae of both pure breeds (Pure Mysore, Nistari, CSR₂ and NB₄D₂) as well as hybrid (♀ Pure Mysore x ♂ CSR₂ and ♀ Nistari x ♂ NB₄D₂) silkworms were used. The haemolymph, midgut and fat body tissues of fifth instar larvae were used for all studies.

2.3.1. Preparation of Haemolymph Sample

The larvae of fifth instar were collected daily from first day till the end of fifth instar, with a regular interval of 24h till the end of fifth instar. The abdominal legs were punctured and the haemolymph was collected in a clean, pre cooled 1.5 ml micro centrifuge tubes containing 1 mM of thiourea, centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C (Mahesha et al., 2000 a & b, 2002) and cell free haemolymph preserved in a deep freezer at -20°C as stock and it was used whenever required.

The haemolymph was diluted twenty times (v/v) with double glass distilled water to estimate amylase, succinate dehydrogenase, non specific esterase as well as alkaline phosphatase activity levels, and it was diluted 250 times to determine the level of total proteins. After appropriate dilution, the samples were centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C, and supernatant was used as the source of protein or enzyme. The samples were kept at 4°C until the commencement of the experiment. For electrophoresis appropriately diluted samples were used.

2.3.2. Preparation of Midgut Tissue Sample:

The midgut tissues was obtained from five larvae daily with a regular interval of 24h till the end of fifth instar by dissecting the larvae in ice cold water and the gut contents were
removed. The tissues were thoroughly washed in sterile distilled water. A 10% (w/v) homogenate of the tissues was prepared in pre cooled sterile distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5°C. The clear supernatant was used for the assay of total proteins, enzymes, and electrophoresis.

2.3.3. Preparation of Fat Body Tissue Sample:

The fat body tissue was obtained from five larvae daily with a regular interval of 24h till the end of fifth instar by dissecting the larvae in ice cold water. The tissue was thoroughly washed in sterile distilled water. A 10% (w/v) homogenate of the tissue was prepared separately in pre cooled distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5°C. The clear supernatant was used for the assay of enzymes, total proteins and electrophoresis.

2.3.4. Estimation of Total Proteins

The total proteins present in haemolymph, midgut and fat body tissues were determined by following the method of Lowry et al., (1951). One ml of the test solution was taken in a clean dry test tube. 5 ml of Lowry’s reagent (98 ml of 2 % sodium carbonate in 0.1 M NaOH + 1 ml of 1 % CuSO4 + 1 ml of 2 % sodium potassium tartarate) was added. After 15 min 0.5 ml of FC (Folin and Ciocalteu’s Phenol) reagent (1:1 diluted with distilled water) was added and left for 30 min under dark condition. The O.D was measured at 660 nm in a UV-Vis spectrophotometer. Bovine serum albumin was used as standard protein. The results were expressed as µg of protein/ µl and µg of protein/mg for haemolymph and, midgut as well as fat body tissue respectively.
2.3.5. Estimation of Amylase Activity:

Quantitative analysis of amylase activity was done in haemolymph, midgut and fat body tissues following the method of Noelting and Bernfeld (1948) using 3, 5-dinitrosalicylic acid reagent, as modified by Ishaaya and Swirski (1976). The reaction mixture contained 2.0 ml of 0.05M sodium phosphate buffer (pH 6.5) and 1.0 ml of freshly prepared 1% starch solution was incubated at 37°C for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph amylase assay and 10 µl tissue (0.5 %) extract for midgut and fat body amylase assay respectively. Incubation of this mixture was carried out for 30 minutes at 37°C in a water bath. After 30 min, 1 ml of 3,5-dinitrosalicylic acid reagent (Solution A: 30 gm of potassium sodium tartrate in 50 ml distilled water + Solution B : 1 gm of dinitrosalicylic acid powder in 10 ml distilled water + 10 ml of 4N NaOH , Solution C: mixing both solutions and making it up to 100 ml ) was added and the mixture was boiled in a water bath for 10 min, then cooled to room temperature. The optical density was measured at 540 nm setting the UV-Vis spectrophotometer to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The activity of the amylase was expressed as µ moles of glucose generated /mg protein/min at 37°C for haemolymph, midgut and fat body tissues.

2.3.6. Estimation of Succinate Dehydrogenase Activity:

Succinate dehydrogenase activity levels were estimated in all the three selected tissues by the method of Nachlas et al., (1960). The reaction mixture contained one ml of sodium phosphate buffer (0.1M, pH 7.4), one ml of 2(4-iodophenyl)-3(4-nitrophenyl) - 5-Phenyltetrazolium chloride (INT,1mg/ml) and one ml of sodium succinate (15mM) was incubated at 37°C for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph amylase assay and 10 µl tissue (0.5 %) extract for midgut and
fat body amylase assay respectively. The tubes were incubated at 37°C for one hour. The reaction was stopped by the addition of 6 ml of glacial acetic acid. The red colour formazan formed was extracted into 6 ml of toluene by keeping the sample tubes overnight at 4°C. The colour was read at 495 nm in a UV-Vis spectrophotometer setting to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The succinate dehydrogenase activity levels were expressed in micromoles of formazan formed / mg protein / min at 37°C for haemolymph, midgut and fat body tissues.

### 2.3.7. Estimation of non specific Esterase Activity:

Quantitative analysis of non specific esterase activity was done in haemolymph midgut and fat body tissues following the method of Valdes and Chambers (1990) with following modifications (Ghasemi Kasmaei and Mahesh 2012 c). The reaction mixture contained 40µl of 0.1M sodium phosphate buffer (pH 7.0), 130 µl double glass distilled water, 20 µl 0.025 M paranitrophenol acetate in acetone was incubated at 37°C for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph esterase assay and 10 µl of tissue (0.5 %) extract for midgut and fat body esterase assay respectively. Incubation of this mixture was carried out for 1 min at 37°C in a water bath. After 1min, 1 ml of ice cold chloroform was added and the contents were mixed thoroughly, then 1.5ml of 0.2M sodium phosphate buffer (pH 9.0) was added. The contents were shaken vigorously in a capped centrifuge tube, centrifuged at 2000 rpm for 5 min in a cooling centrifuge at 5°C. The supernatant was collected and the optical density was measured at 400 nm setting the UV-Vis spectrophotometer to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The activity of the enzyme was expressed as µmoles of paranitrophenol released /mg protein/min at 37°C. Paranitrophenol was used as standard.
2.3.8. Estimation of Alkaline Phosphatase Activity:

Quantitative analysis of alkaline phosphatase activity was done in haemolymph, midgut and fat body tissues. The reaction mixture contained 1ml of 0.1M sodium carbonate buffer (pH 10) containing 50 mM paranitrophenol phosphate was incubated at 37°C for 5 min. After this pre incubation, appropriately (1:10) diluted 10 µl haemolymph for haemolymph alkaline phosphatase assay and 10 µl tissue (0.5 %) extract for midgut and fat body alkaline phosphatase assay respectively. Incubation of this mixture was carried out for 30 min at 37°C in a water bath. After 30 min, 2 ml of 0.1 N NaOH was added and the contents were mixed thoroughly. Then the volume was made up to 4 ml with buffer. The contents were shaken vigorously and the optical density was measured at 540 nm setting the UV-Vis spectrophotometer to zero with blank consisted of incubation mixture to which enzyme sample was added after termination of the reaction. The activity of the enzyme was expressed as µmoles of paranitrophenol released /mg protein/min at 37°C. Paranitrophenol was used as standard.

2.3.9. Estimation of Deoxyribonucleic Acid:

A 10% homogenate was prepared in buffered saline (0.15 M Nacl and 0.15 M sodium citrate, pH 7.0). Homogenate was centrifuged at 8,000 rpm for 15 minute and resulting supernatant was taken for estimation of DNA (Gracy, 2012) content. The amount of DNA was estimated by diphenylamine method (Schneider, 1957). To 1 ml of sample in a clean dry test tube 4 ml of diphenylamine reagent (1% diphenylamine in glacial acetic acid + 0.25% concentrated sulphuric acid) was added and incubated in a boiling water bath for 10 min. After 10 min the contents were cooled to room temperature and the optical density was read at 595 nm setting the UV-Vis spectrophotometer to zero with blank containing distilled water.
in place of sample. Calf thymus DNA was used as standard. The results were expressed as µg of DNA /mg tissue.

2.3.10. Estimation of Ribonucleic Acid:

A 10% homogenate was prepared in buffered saline (0.15 M Nacl and 0.15 M sodium citrate, pH 7.0). Homogenate was centrifuged at 8,000 rpm for 15 minute and resulting supernatant was taken for estimation of RNA (Gracy, 2012) content. The concentration of RNA was estimated by orcinol method (Schneider, 1957). To 1 ml of sample in a clean dry test tube 3 ml of orcinol reagent (0.1% ferric chloride in concentrated HCl + 3.5 ml of 6% orcinol in alcohol) was added and incubated in a boiling water bath for 20 min. After 20 min the content was cooled to room temperature and the optical density was read at 665 nm setting the UV-Vis spectrophotometer to zero with blank containing distilled water in place of sample. Yeast RNA was used as standard. The results were expressed as µg of RNA /mg tissue.

2.3.11. Statistical Analysis:

The experimental data were statistically analyzed through SPSS by one way ANOVA (Fisher and Yates 1953) and Scheffe’s post hoc test (Scheffe, 1959) wherever they were applicable. The regression analysis was used to determine the biomolecule-commercial traits relationship between different biomolecules and expression of commercial characters using the equation Y = bx + a.

2.3.12. Qualitative analysis of total proteins by Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis:

The Qualitative analysis of total proteins was carried out in haemolymph according to Laemmli (1970) in Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-
PAGE) with the discontinuous buffer system with slight modifications (Mahesha et al., 2000). The vertical slab gel apparatus (Chromous Biotech, India) was used.

a) Sample Preparation:

The haemolymph as well as tissue extracts collected was mixed with nine volumes of 0.025 M Tris-HCl buffer (pH 6.8), containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.006% bromophenol blue, and then centrifuged at 3000 rpm for 5 minutes. The supernatant was incubated in boiling water bath for 5 min. After bringing to room temperature, it was used as sample solution.

b) Preparation of running and stacking gels:

The gels measuring about 17.5 cms x 19 cms and 0.2 cms thickness were polymerised. A 12% separating gel was prepared by mixing 9 ml of 1.5 mM Tris-HCl buffer (pH 8.8), 14.4 ml of 30% acrylamide in 0.8% bis-acrylamide, 36 μl of N,N,N’-N’-tetramethylenediamine (TEMED), 360 μl of 10% SDS, 11.82 ml of distilled water and 360 μl of 10% ammonium persulphate. The gel was then overlayed with water-saturated butenol in order to achieve even surface so that polymerisation occurs within 30-45 minutes at room temperature. A 5% stacking gel of 2 cm deep was prepared by mixing 4.5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 3 ml of 30% acrylamide in 0.8% bis-acrylamide, 18 μl of TEMED, 180 μl of 10% SDS, 7.122 ml of distilled water and 180 μl of 10% ammonium persulphate. The comb was inserted carefully by avoiding air bubbles, so that polymerization occurs within 30 minutes at room temperature.

The reservoir buffer was prepared by dissolving 3.03gm of Tris, 14.4gm of glycine, 10 ml 10% SDS in enough quantity of distilled water, and finally, volume was made upto 1000 ml (pH 8.3).
c) **Electrophoresis run:**

Pre-electrophoresis was carried out at 20°C supplying a constant current of 1.5 mA per slot for 20 minutes. A uniform quantity of protein (100 µg) from each extract was loaded to each slot of the gel. Molecular weight markers from Genei were also used in a slot to compare the molecular weight of the proteins separated from that of the sample. The molecular weight markers consisted of Myosin (205 kDa), Phosphorilase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (43 kDa) Carbonic Anhydrase (29 kDa), Soybean Trypsin inhibitor (20.1 kDa), Lysozyme (14.3 kDa), Aprotinin (6.5 kDa) and Insulin (3 kDa).

After loading the samples, electrophoresis was carried out by supplying a constant current of 2 mA per slot for stacking zone and 3 mA per slot for separating zone at 20 ºC. The run was stopped when the front reached the bottom of running gel.

d) **Gel fixing and staining:**

The gels, soon after the removal, was fixed in 12% trichloroacetic acid for 30 minutes and then in 10% acetic acid for 30 minutes. After fixation, the gels were stained in 0.3% Coomassie brilliant blue R-250 in a mixture of water, methanol and acetic acid (1:1:1; v/v) for overnight.

e) **Gel destaining, storing and scanning:**

The gels were destained for 6-8 hours in a mixture of water, methanol and acetic acid (6:3:1; v/v) and then stored in 10% acetic acid with 5% glycerol. The gels were scanned and photographed in a gel scanner (Vilber Lourmat Bioprofil image analysis system).

2.3.13. Qualitative analysis of isozymes by – Native Poly Acrylamide Gel

**Electrophoresis:**

The qualitative analysis of amylase, Succinate dehydrogenase, non specific esterase and alkaline phosphatase were carried out using the selected tissue samples in Poly
Acrylamide Gel Electrophoresis (PAGE) with the discontinuous buffer system. The vertical slab gel apparatus (Chromous Biotech, India) was used.

a) Sample Preparation:

The tissue sample collected was mixed with equal volumes of 0.5 M Tris-HCl buffer (pH 6.8), 10% glycerol and 0.006% bromophenol blue, and then centrifuged at 3000 rpm in a cooling centrifuge at 5 °C for 5 minutes. The clear supernatant was used as sample solution.

b) Preparation of running and stacking gels:

The gels measuring about 17.5 cms x 19 cms and 0.2 cms thickness were polymerised. A 8% separating gel was prepared by mixing 9 ml of 1.5 mM Tris-HCl buffer (pH 8.8), 9.6 ml of 30% acrylamide, 36 µl of N, N, N’-N’-tetramethylenediamine (TEMED), 17 ml of distilled water and 360 µl of 10% ammonium persulphate. The gel was then overlayered with water-saturated butenol in order to achieve even surface so that polymerization occurs within 30-45 minutes at room temperature. A 4% stacking gel of 2 cm deep was prepared by mixing 4.5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 2.4 ml of 30% acrylamide, 18 µl of TEMED, 11 ml of distilled water and 180 µl of 10% ammonium persulphate. The comb was inserted carefully by avoiding air bubbles, so that polymerization occurs within 30 minutes at room temperature.

c) Reservoir buffer:

The reservoir buffer for Amylase activity: This was prepared by dissolving 6.05g of Tris, 28.80g of glycine in enough quantity of distilled water, and finally, volume was made up to 1000 ml (pH 8.2 – 8.4).

The reservoir buffer for Succinate dehydrogenase, esterase and alkaline phosphatase activity: This was prepared by dissolving 3g of Tris, 14.4g of glycine in enough quantity of distilled water, and finally, volume was made up to 1000 ml (pH 8.2 – 8.4).
d) Electrophoresis run:

Pre-electrophoresis was carried out at 5ºC supplying a constant current of 1.5 mA per slot for 20 minutes. A uniform quantity of protein (150µg) from each extract was loaded to each slot of the gel. After loading the samples, electrophoresis was carried out by supplying a constant current of 2 mA per slot for stacking zone and 3 mA per slot for separating zone. The run was stopped when the front reached the bottom of running gel.

e) Activity Staining:

2.3.14. Amylase:

The gels, soon after the removal, washed in running distilled water followed by the incubation in 1% freshly prepared starch solution at 37º C in a rotary shaker for 30 min. After incubation the gel was placed in Iodide solution (5 mM I$_2$-KI in distilled water) for 15 min or until negative bands appeared. Then the gels were scanned, analysed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

2.3.15. Succinate dehydrogenase:

The gels, soon after the removal, washed in running distilled water followed by the incubation in 100 ml of sodium phosphate buffer (50mM pH 7.0) containing sodium EDTA 400 mg, sodium succinate 250 mg, ATPNa$_2$ 50mg, NAD$^+$ 70 mg, NBT 40mg and PMS 2mg at 37ºC in rotary shaker in dark for 1 h or until the bands appeared. Then the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

2.3.16. Non Specific esterase:

The gels, soon after the removal, washed in running distilled water and incubated in the following solution C in a rotary shaker at 37ºC in dark for 20 min or until the bands appeared. After the appearance of bands the reaction was stopped by the addition of 2-3 ml
glacial acetic acid. After the appearance of bands, the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

**Solution A** was prepared by dissolving 25 mg α naphthyl acetate and 25 mg β naphthyl acetate in 1 ml of acetone followed by the addition of 1 ml water and 12.5 ml of 0.5 M sodium phosphate buffer pH 5.9.

**Solution B** was prepared by dissolving 25 mg fast blue RR salt in 2 ml of solution A followed by the addition of 12.5 ml 0.1 M sodium phosphate buffer pH 6.5.

**Solution C** was prepared by mixing solution A and B.

**2.3.17. Alkaline Phosphatase:**

The gels, soon after the removal, washed in running distilled water followed by the incubation in 100 ml of Tris-HCl buffer (50mM pH 8.5) containing polyvinyl pyrolidone 500 mg, fast blue RR salt 100 mg, sodium alpha naphthyl phosphate 100mg, magnesium chloride 60 mg, manganese chloride 60 mg and sodium chloride 2g at 37°C in rotary shaker in dark for 1 h or until the bands appeared. Then the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

**2.3.18. Isolation of genomic Deoxyribonucleic acid:**

Each individual larva at fifth instar (day 3) was collected and DNA was isolated from the midgut tissue (Suzuki *et al.*, 1972; Thanananta *et al.*, 1997; Nagaraja, 2002; Nagaraja and Nagaraju, 1995). About 100 mg of the midgut tissues were dipped in fixing solution and absolute alcohol for 30 min. After allowing alcohol to evaporate, the tissue was ground with a mortar and pestle and the homogenized tissue was transferred to pre-warmed extraction buffer (100 mM Tris buffer, pH 8.0 containing 1.4 M NaCl, 25 mM EDTA, pH 8.0, 2.5% SDS and 0.8% mercaptoethanol) in an Oakridge tube. It was incubated at 65°C in a water bath and occasionally mixed by gentle swirling. After one hour of incubation, it was removed from the water bath. The DNA was then extracted with chloroform-isoamyl alcohol (24:1).
The extracted DNA was precipitated in ethanol in the presence of 3.0 M sodium acetate (pH 5.4 -5.6) and resuspended in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). The RNA contamination was removed by incubating with RNase A (100 mg/ml) at 37°C for 1 h. The genomic DNA thus obtained was quantified on 1% agarose gel.

2.3.19. Random Amplification of Polymorphic Deoxyribonucleic acid Analysis:

Genomic DNA isolated was PCR amplified under the following conditions. DNA 1 µl, RAPD primer 2 µl, dNTPs(10mM) 2 µl, Assay buffer (10X) 5 µl, Taq DNA Polymerase 0.5 µl, Water 39.5 µl. Total volume was 50 µl. The PCR conditions included initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min with final extension at 72 °C for 2 min. Polymorphic RAPD Primer Used in the Study was 5’-AGGACTCGATAACAGGCT-3’. The PCR products were loaded on 2 % agarose gel for checking. 100 bp Ladder contains 10 DNA fragments of size 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp and 1kb was used as a molecular weight marker (Chromous Biotech, India).

2.3.20. RAPD analysis software details:

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000. For estimating the sampling distribution by resampling with replacement from the original sample Bootstrapping was used. In making phylogenetic trees, the approach is to create a pseudo alignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudo alignment will be as long as the original alignment and will be used to create a distance matrix and a tree.