CHAPTER III
MATERIALS AND METHODS

Crop improvement through selection and breeding have been well adopted worldwide and given priority for developing the new varieties that can possess all the possible desired traits to increase the yield potential, resistance to disease and insect pests, bold beans, superior cup quality and wider adaptability (Van der Vossen, 2001). Utilization of genetic resources has played a major role in identification of desired genotypes for crop improvement through hybridization in combination with suitable statistical procedures (Sera, 2000). In the present study, similar recommended materials and methodology were adopted to carry out the genetic analysis and molecular characterization of F1 hybrids derived from various crosses of station-bred released selections.

3.1. Location of the experiment and altitude

The study was carried out during 2008-09 and 2009-10 on fifteen F1 progenies derived from Dwarf x Tall, Tall x Dwarf and Tall x Tall crosses established during 1997-98 at Coffee Research Sub Station, Chettalli, Kodagu District, Karnataka, India. Kodagu is one of the smallest district in the southern part of Karnataka covering an area of 4,104 Sq Km. It is also known as Coorg. The holly river Cauvery has its origin in this district, which divides the district into two parts; one is North Kodagu and other part South Kodagu. North Coorg is predominantly arabica growing area while South Kodagu is known for good quality robusta production. This is the highest coffee producing district in Karnataka. Coorg is very famous for orange variety called as Coorg mandarin besides its good quality of Pepper and Cardamom production in this district.

Coorg lies between the latitude 11º55' and 12º50' N and longitude 75º25' and 76º40' E. The elevation ranges from 750-1100 metres above MSL. This Research Station is located at an elevation of about 900 metres with gentle slope.
3.2. Weather

The average annual rainfall in Kodagu is around 1000 to 2500 mm. The temperature range during winter is from 15 to 20 °C while 25 to 28 °C during summer. Fall in the temperature has been experienced up to 9 °C during the month of December to February. Nature has blessed this district, with thick forest area with Sandal wood, Rose wood and numerous species of trees and orchids. The Coffee Research Station receives an average annual rainfall of 1500 mm, temperature range of 10 to 30 °C and 40 to 90 percent R.H.

3.3. Soils

The soil is red laterite, acidic with high level of organic carbon with the pH range of 6.0 to 6.5 in the experimental block.

Table: 3.1. The soil profile of CRSS, Chettalli during the study period

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Particulars</th>
<th>Observed values</th>
<th>Optimum requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Soil pH</td>
<td>5.50 – 6.00</td>
<td>6.20</td>
</tr>
<tr>
<td>2.</td>
<td>Organic Carbon (%)</td>
<td>0.25 – 2.50</td>
<td>0.50 – 2.50</td>
</tr>
<tr>
<td>3.</td>
<td>Available Phosphorus (kg/ha)</td>
<td>9.00 – 60.00</td>
<td>10.0 – 22.00</td>
</tr>
<tr>
<td>4.</td>
<td>Available Potassium (kg/ha)</td>
<td>115.00 – 260.00</td>
<td>126.00 – 250.00</td>
</tr>
</tbody>
</table>

3.4. The experimental materials

The details about the parent cultivars used in hybridization are as follows:

Table: 3.2. Coffee cultivars utilized as parental lines

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parents</th>
<th>Sl. No.</th>
<th>Parents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S.795 (Kents x S.288)</td>
<td>7</td>
<td>Sln.9 ( HDT x Tafarikela)</td>
</tr>
<tr>
<td>2</td>
<td>S.881 (Rume Sudan wild arabica)</td>
<td>8</td>
<td>S.4179 (Catimor x Catuai)</td>
</tr>
<tr>
<td>3</td>
<td>Tafarikela (Ethiopian collection)</td>
<td>9</td>
<td>Sln.11 (Natural hybrid of liberica x eugeniodes)</td>
</tr>
<tr>
<td>4</td>
<td>Devamachy hybrid (A natural hybrid of Arabica x Robusta)</td>
<td>10</td>
<td>Sln.12 (Caturra x HDT) Catimor known as Cauvery</td>
</tr>
<tr>
<td>5</td>
<td>Sln.5B (S.333 x Devamachy hybrid)</td>
<td>11</td>
<td>CxR (Congensis x Robusta)</td>
</tr>
<tr>
<td>6</td>
<td>Sln.6 (Arabica x Robusta)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Parental Cultivar “Cauvery”

Plate: 1. Dwarf type cultivar with compact bush habit
Plate: 1a Closely situated fruit clusters with normal fruit size

Parental Cultivar: S.4179 (Catimor x Catuai)

Plate: 2 Dwarf type and compact bush habit
Plate: 2a Closely situated fruit clusters with normal size fruit
3.4.1. Characteristic features of the parent cultivars

Important characteristic features of the individual parents involved in hybridization programme are as follows:

1. **S.795 (S.288 x Kents arabica)** - It is popularly known as S.795 with tall type bush behaviour, high yield potential and better cup quality in spite of severe incidence of leaf rust. This cultivar was used to cross with Sln.9 to develop the progeny that could retain the high yield potential, bold bean size, superior cup quality and higher degree of tolerance to the common leaf rust races.

2. **S. 881 (Rume Sudan wild arabica)** - Tall type and compact bush, thick stem, smaller narrow leaves, shorter inter nodes as compared to other tall arabicas, medium sized fruits (mostly ‘B’ grade beans) with raised naval, loose fruit clusters, moderate yielder, plant with horizontal resistance to leaf rust (Sreenivasan *et al.*, 1993), light bronze tipped young leaves with quality FAQ and above. To obtain a progeny with semi-dwarf bush spread suitable for higher density planting, higher yield than Catimor (Cauvery), with 60-65 percent ‘A’ grade beans, superior beverage quality, S. 881 was crossed with cultivar “Cauvery”. Introduction of horizontal resistance in the progeny was one of the important goals of the hybridization.

3. **Sln.4 (Tafarikela)** - In general, Tafarikela is one of the tall type Ethiopian varieties characterized by compact bush, medium inter-nodal length, coppery leaf-tip colour, horizontal rust resistance and drought tolerance. Medium sized fruits with 40-45 percent of ‘A’ grade beans, loose fruit clusters, high yielder, early ripener, FAQ and above cup quality. An outstanding plant with high yield (25.0 kg ripe cherries plant⁻¹) complete rust resistance with normal fruit size was identified in a Tafarikela plot established at CRSS, Chettalli. But this plant showed higher content of fruit floats (5.0 kg out of 25.0 kg ripe fruits) which is an undesirable character of the good cultivar. Realization of even 20.0 kg
fruit plant$^1$ would produce around 7-8 tones clean coffee ha$^{-1}$. Along with high yielding potential of this plant, introgression of the genes governing the horizontal resistance was also one of the major objectives of crossing Tafarikela with “Cauvery” cultivar so as to develop a cultivar having semi-dwarf compact bush like Cauvery combined with vertical and horizontal rust resistance, higher productivity and superior quality.

4. **Sln.5 (Devamachy hybrid)** - This is a natural Robusta x Arabica hybrid spotted in Devamachy, a coffee estate in Coorg (Srinivasan, 1996) having tall type, thick and sturdy branches, broad-roundish lathery leaves with bronze tipped young leaves, drought hardy, leaf rust tolerant, poor yielder with abnormal fruits (mostly pea berries), tight fruit clusters like robusta. This plant was used in crossing with ‘Cauvery’ to introduce drought hardiness, tight clusters and robusta genes for leaf rust resistance in the progeny (Sreenivasan *et al.*, 1993).

5. **Sln.5B (S.333 x Devamachy hybrid)** - This is a tall type cultivar with high degree of resistance to leaf rust (Sreenivasan *et al.*, 1993) and moderate to high yielding behavior even up to 2500 kg ha$^{-1}$ in Pulney Hills. It has proven record of higher production and good cup quality characteristics at RCRS, Thandigudi, in Tamil Nadu. An accession no. S.4422 is the individual plant progeny of Sln.5B (S.2931) planted at “Pillavalli” a private coffee estate in Pulney Hills and it was established at CRSS, Chettalli during 1991. It has acquired all the desirable traits and found superior to that of Sln.5B regarding yield and rust resistance. In order to obtain a genotype above the normal level of yield, durable resistance to leaf rust, compact bush and good bean and liquor quality, S.4422 was hybridized with ‘Cauvery’ cultivar.
Parental Cultivar: S.795 (Kents x S.288)

Plate: 3 Tall type bush with long internodes
Plate: 3a Loose fruit clusters, normal fruit size

Parental Cultivar: S.881 (Rume Sudan Wild Arabica)

Plate: 4 Tall type bush with long internodes and narrow leaves
Plate: 4a Loose clusters with small fruits
6. **Sln.6 (Robusta x Arabica)** - It is an inter-specific hybrid of tall type bush developed at CCRI with an objective to incorporate robusta genes for rust resistance in arabica hence, robusta was crossed with Kents arabica to serve the purpose. It has recorded high rust resistance upto 85 percent (Sreenivasan *et al.*, 1993) and production potential with more than 60 percent ‘A’ grade beans FAQ cup quality. Since, this selection possesses high level of rust resistance and desirable quality; it was bred with cultivar ‘Cauvery’ to transfer these genetic traits to the progeny of semi-dwarf character inherited from ‘Cauvery’.

7. **Sln-9 (HDT x Tafarikela)** – The general characteristics of this cultivar are Tall type bush span, thick stem girth, longer internodes, broader leaf size than the other arabicas, bronze color young leaf tip, bold size fruits with 12 percent fruit floats, loose fruit clusters, moderate yielder (1250 kg clean coffee ha$^{-1}$ with consistency in yield), 70 percent ‘A’ grade beans. Beside these traits, it shows field tolerance to leaf rust to an extent of 70 percent. The plant chosen from the population had all those characters that pertain to Sln.9 except the green color of leaf tip and protruded fruit naval as against normal one and had complete resistance to leaf rust and with good cup quality (above FAQ).

8. **S.4179 (Catimor x Catuai)** - A semi-dwarf bush with shorter inter-nodal length and green tipped young leaves, comparatively higher tolerance to leaf rust (60-70 percent) than Cauvery (5-10 percent), moderate yielder with 1115 kg ha$^{-1}$ (7years average), medium sized bush, drought susceptible, normal fruit size, loose fruit clusters and fair average cup quality (FAQ) (Anonymous, 2002)

9. **Sln.11 (A spontaneous tetraploid mutant of C. liberica x C. eugenioides hybrid)** - This selection was evolved through accession no. S. 2464, a hybrid of C. liberica x C. eugenioides (Narasimhaswamy and Vishveshwara, 1967). It is a tall type plant material with long internodes, narrow leaf breadth, bronze leaf tip color, leaves resemble arabica
and possess good resistance to leaf rust, good bearing capacity but low productivity due to smaller bean size with 15 to 20 percent ‘A’ grade beans and it has comparatively lower cup quality to the other arabica cultivars (Anonymous, 2003). This cultivar was selected as a male parent to introduce liberica rust resistance genes in the progeny combining the genes for bold bean, rust resistance and cup characteristics from Sln.9 female parent.

10. Sln.12 (Catimor) - A semi-dwarf bush with shorter inter-nodal length and green tipped young leaves, high yielder, medium sized bush, drought susceptible, normal fruit size, loose fruit clusters and fair average cup quality (FAQ). This selection was released by the CCRI during 1985 as “Cauvery”. In the beginning, it had resistance to leaf rust but after a period of five years became susceptible and hence decided to be breed out a progeny that could acquire compact bush like ‘Cauvery’, better resistance to leaf rust bold size beans like Sln.9, moderate yield and superior cup performance. Considering Sln.9 as one of the parents was the introgression of horizontal resistance character transmitted from Tafarikela to Sln.9.

11. C x R (Congensis x Robusta) - Compact bush with drooping branches, long internodes, smaller leaf size with narrow leaf breadth as compared to robusta, tight fruit clusters, bold fruits with protruded navel, 50-55 percent ‘A’ grade beans, high yielding 2.0-2.5 MT ha⁻¹ with good cup quality above FAQ and leaf rust resistant.

3.4.2. The F₁ progenies

The above F₁ hybrid population derived from the various arabica crosses was planted at a spacing of 6′ x 5′ in a plot established at CRSS, Chettalli and used for the present study. The F₁ hybrids from all the crosses were maintained in the same environmental conditions with uniform shade and normal agronomical practices. All the experimental plant materials were nurtured under the normal level of nutritional application.
Parental Cultivar: Tafarikela (Ethiopian Variety)

Plate: 5 Tall type bush, long internodes and normal leaf size

Plate: 5a Loose clusters with medium size fruit

Parental Cultivar: Devamachy Hybrid

Plate: 6 Tall type bush with broader leaves

Plate: 6a Loose clusters and abnormal size fruits
3.5. Experimental method

The observations on morphological parameters viz; stem girth, plant height, number and length of primary, number and length of secondary, number of flowering and total nodes, inter-nodal length and fruits per cluster were recorded. Data were subjected to statistical analysis to assess the significance of differences in performance between the hybrids. Genotypic and phenotypic variances, co-efficient of variation, heritability in broad sense ($h^2$) and narrow sense ($H^2$) and correlation co-efficient between the morphological characteristics were calculated based on the procedure followed by Srinivasan et al., (1979).

Table: 3.4. The F1 progenies of different parental cross combinations used for the study

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Dwarf x Tall</th>
<th>Sl. No.</th>
<th>Tall x dwarf</th>
<th>Sl. No.</th>
<th>Tall x Tall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S.4842 (Cauvery x Sln.9)</td>
<td>6</td>
<td>S.4852 (Sln.9 x Cauvery)</td>
<td>11</td>
<td>S.4856 (Sln.9 x Sln.11)</td>
</tr>
<tr>
<td>2</td>
<td>S.4845 (Cauvery x S.881)</td>
<td>7</td>
<td>S.4854 (S.881 x Cauvery)</td>
<td>12</td>
<td>S.4857 (Sln.5B x Sln.9)</td>
</tr>
<tr>
<td>3</td>
<td>S.4848 (Cauvery x Devamachy)</td>
<td>8</td>
<td>S.4860 (Sln.5B x Cauvery)</td>
<td>13</td>
<td>S.4859 (Sln.5B x S.795)</td>
</tr>
<tr>
<td>4</td>
<td>S.4855 (Cauvery x Tafarikela)</td>
<td>9</td>
<td>S.4876 (Sln.6 x Cauvery)</td>
<td>14</td>
<td>S.4861 (Sln.6 x S.795)</td>
</tr>
<tr>
<td>5</td>
<td>S.4864 (Cauvery x Triploid CxR)</td>
<td>10</td>
<td>S.4863 (Tafarikela x S.4179)</td>
<td>15</td>
<td>S.4875 (Sln.6 x Sln.9)</td>
</tr>
</tbody>
</table>

Data on morphological characters such as bush spread, stem girth, primary thickness, intermodal length, leaf length and breadth and number of fruits per cluster, were recorded in four replications using seven plants per replication.

Similarly, fruit length and breadth were also observed. Three samples were drawn from the lot of each progeny to determine 100 bean weight of ‘A’ grade size and cup quality. Experiment was conducted following randomized block design (RBD) and analyzed statistically. The coffee samples representing all fifteen F1 populations were prepared through wet processing method and cup quality was evaluated by the Quality Control Division, Coffee Board, Bangalore. The scores awarded for each quality parameters viz; Aroma/Fragrance, Body, Acidity, Mouth feel, After taste, Flavour and Overall were subjected to correlation analysis.
Parental Cultivar: Sln.5B (S.333 x Devamachy Hybrid)

Plate: 7 Tall type bush, with long internodes and normal leaves

Plate: 7a Tight clusters and normal size fruits

Parental Cultivar: Sln.6 (Swarnagiri)

Plate: 8 Tall type bush, with long internodes and normal leaf size

Plate: 8a Tight clusters with normal size fruits

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Table: 3.5. Name of the parameters recorded during the period of experimentation

<table>
<thead>
<tr>
<th>Growth Parameters</th>
<th>Yield parameters</th>
<th>Outturn ratio</th>
<th>Bean parameters</th>
<th>Quality parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bush spread (cm)</td>
<td>1. No. of fruits/cluster</td>
<td>1. Fruit : wet parchment</td>
<td>1. 100 bean wt. (gm)</td>
<td>1. Cup characteristics (cup testing)</td>
</tr>
<tr>
<td>2. Stem girth (cm)</td>
<td>2. Fruit length (cm)</td>
<td>2. Fruit: dry parchment</td>
<td>2. Bean length (cm)</td>
<td>2. Caffeine content in raw beans (percent)</td>
</tr>
<tr>
<td>3. Primary thickness (cm)</td>
<td>3. Fruit breadth (cm)</td>
<td>3. Dry parchment: clean coffee</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Primary’s internodal length (cm)</td>
<td>4. 100 fruit wt. (gm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Number of secondary shoots</td>
<td>5. 100 fruit vol. (ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Leaf length (cm)</td>
<td>6. Fruit floats (percent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Leaf breadth (cm)</td>
<td>7. Yield/plant (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. L:B ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Leaf area (cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the F₁ crosses were grouped into 3 classes namely, Dwarf x Tall, Tall x Dwarf and Tall x Tall and each class possesses 5 cross combinations. The result is presented in tabular form separately for each group of 5 crosses. Similarly, the results on cup quality and its relation with morphological characters were exhibited graphically.

3.6. Estimates of genetic components

3.6.1. Genotypic and phenotypic variances and its coefficient of variations

Creation of genetic variability and selection for important traits is crucial activity that every plant breeder needs its application to achieve better yield and other agronomic traits. Variation is the basis for plant breeding and it is the occurrence of differences between the individuals due to variation in their genetic composition and/or the environment in which they are grown (Falconer and Mackay, 1996; Singh, 2000).

*C. arabica* L. presently cultivated in India and abroad has mainly two varieties known as Bourbon and Typica (arabica) coffee. It is believed that the other cultivated forms known as varieties are the mutants of these two varieties of arabica.
Parental Cultivar: Sln.9 (HDT x Tafarikela)

Plate: 9 Tall type bush, long internodes and bigger leaves

Plate: 9a Loose clusters and bold size fruits

Parental Cultivar: Sln.11 C. Liberica x C. eugenenoides

Plate: 10 Tall type bush, long internodes and narrow leaves

Plate: 10a Loose cluster and small size fruits
A wide range of variability in the cultivated forms of arabica has been reported for shape and size of plant architecture, leaf, branches, internodes, fruit, stem, bean, root, liquor quality etc. Carvalho et al., (1991) observed more than 40 varieties of arabica with single gene mutation and they are highly variable in characteristics such as caffeine content, leaf shape and color, type of flower and blossom behavior, fruit and seed colour, resistance to diseases and insects and nematodes. This implies the potential for utilizing such variability in crop improvement programmes. Although an estimate of genetic and phenotypic variability is often considered as a pre-requisite for initiating appropriate breeding procedures, no previous reports on the F1 populations of arabica crosses are available in India. Therefore, the current study was undertaken with the aim of estimating genetic variability, heritability and genetic advance in F1 hybrids derived from the various crosses of arabica. The following formula was used for estimation of the genetic parameters. The magnitudes of components of variances have been obtained from analysis of variance to estimate the different genetic parameters as described by Atherly et al., (1999) and Singh, (2000).

\[ V_g = V_p - V_e \]
\[ V_p = V_g + V_e \]

Where, \( V_g \), \( V_p \), and \( V_e \) are genotypic variance, phenotypic variance and environmental variance respectively.

Genotypic and Phenotypic coefficients of variation were calculated according to the method of Burton de Vane (1953).

Genotypic coefficient of variation \( GCV = \frac{\sigma_g \times 100}{\bar{X}} \)

Where, \( \sigma_g \) is genotypic standard deviation and \( \bar{X} \) is grand mean of the genetic component.

Phenotypic coefficient of variation \( PCV = \frac{\sigma_p \times 100}{\bar{X}} \)

Where, \( \sigma_p \) is phenotypic standard deviation.
Parental Cultivar: C x R \((C. \text{canephora} \times C. \text{congensis})\)

Plate: 11 Tall type, drooping branches, long internodes and broader leaves

Plate: 11a. Tight clusters, bold fruits and protruded navel

Parent plant material crossed with ‘Cauvery’

Plate: 11b A triploid sterile plant \((3n=33)\) used as male parent for interspecific crossing with ‘Cauvery’ cultivar

Plate: 11c A branch of triploid plant with broad and lathery leaves
3.6.2 Genetic advance (G.A.)

Phenotype is an outcome of genotypic and environmental interaction hence, phenotypic superiority of the selected plants or family over the base population is not mainly because of their genetic advancement. Genetic advance is an improvement in the mean genotypic value of the selected group over the base population. For homogeneous population heritability in broad sense is used whereas for heterogeneous population heritability in narrow sense is more realistic for estimation of genetic advance. To carry out effective selection, the information on genetic variation among coffee genotype, the nature of component traits on which selection would be effective and influence of environmental factors on each trait to be known. Thus effective selection not only depends on estimation of genetic variation but also on the proportion of heritable variation and expected genetic gain that would be obtained. Characters not greatly influenced by the environment usually have a high heritability.

This may influence the choice of selection procedure opted by the plant breeder to decide which selection method would be most advantageous to improve the character to predict gain from selection and to determine the relative importance of genetic effects (Poehlman and Sleper, 1995). In the present study, the estimates of Genetic advance and Heritability in broad sense were calculated based on the formula described by D.B. Singh, (1999) for F1 population studied in the experiment. The formula used is as follows:

\[ Gs = (k) (\sigma_p) (H) \]

Where, \( Gs \) is the genetic advance,

- \( (k) \) is selection differential
- \( \sigma_p \) is phenotypic standard deviation and
- \( H \) is heritability in narrow sense

Percentage of genetic advance was calculated based on the formula indicated here under

Percent Genetic advance (\( Gs \)) = \( Gs/\mu \times 100 \) where, \( \mu \) is the mean of the base population
F<sub>1</sub> PROGNEY OF DWARF x TALL CULTIVARS

Plate: 12 S.4842 (Cauvery x Sln.9) with medium bush spread

Plate: 12a Normal size fruits on medium internodes

F<sub>1</sub> PROGNEY OF DWARF x TALL CULTIVARS

Plate: 13 S.4845 (Cauvery x S.881) with medium bush spread

Plate: 13a Normal size fruits on medium internodes
3.6.3. Heritability

Heritability estimates serve as an important tool to the breeders to understand the degree of variation caused due to genotypic (broad sense heritability) or additive (narrow sense heritability) effects. If heritability of a character is very high (0.8 or more), selection for such character becomes easier. Similarly, when the heritability of a trait is lower than 0.4 selection of the plant material would be rather difficult or unworthy.

In the present study, heritability in broad sense \( (h^2) \) was calculated to find out the variation caused by the genotypes with the help of following formula (Chaudhary, 1982).

\[
h^2 \text{(bs)} = \frac{V_g \times 100}{(V_g + V_e)}
\]

Whereas, \( V_g \) denotes genotypic variance and \( V_e \) represents environmental component of variance.

3.7. Correlation of characters

The quantitative traits association between genotype and phenotype is a very complex subject and not yet completely understood by the biological scientists because; neither genotype nor phenotype as such is transmitted to the offspring from one generation to the next generation. But what is transferred is invisible cluster of genes from both of the parents at the time of fusion between the paternal and maternal gametes. Under the influence of prevailing environment, embryonic development begins and come into a shape of measurable phenotype that provides passage to the breeders to locate the genetic basis for emergence of several qualitative and quantitative phenotypic characters (Naraian, 1993).

The quantitative traits are governed by the polygenic action, expression of one character not only depends one factor but on several genetic factors. In this context, the genetic behaviour and correlation between the characters play a crucial role in plant breeding programme.
F₁ PROGENY OF DWARF x TALL CULTIVARS

Plate: 14 S.4847 (Cauvery x Devamachy hybrid) with medium bush spread

Plate: 14a Normal size fruits on medium internodes

F₁ PROGENY OF DWARF x TALL CULTIVARS

Plate: 15 S.4855 (Cauvery x Tafarikela) with medium bush

Plate: 15a Normal size fruits on medium internodes
3.8 Heterosis

The word ‘Heterosis’ denotes the superiority of a F1 hybrid over both of its parents for yield, plant vigour, and some other important characters (Singh, 1999). The difference between the hybrid and the mean of the two parents is usually called midparent heterosis. High parent heterosis is preferred in some circumstances, particularly in self-pollinated crops, for which the goal is to find a better hybrid than either of the parents (Falconer and Mackay, 1996). The considerable cases of heterosis have been reported in coffee too where, two parents of different origins were crossed to produce F1 progeny. This was due to the effects of complementary epistatic genes for yield. Srinivasan and Vishveshwara (1978) has observed heterosis in arabica hybrids and suggested for better exploitation of F1 hybrids as ‘F1 Hybrid varieties’ for commercial cultivation of coffee.

They observed the additive genes effects that caused the variation in the bean size and cup quality characters. Midparent value could well be used to predict the performance of the hybrid lines for these characters. In the present course of study, the parents involved for breeding the F1 hybrids, some are from different origins and genetic backgrounds which provide the ample chances for heterosis in the F1 population used in the present study. The heterosis was determined based on the following formula demonstrated by Chaudhary (1982):

\[
\text{Heterosis} = \frac{F_1 - \text{Mid parent value}}{\text{Mid parent value}} \times 100
\]

\[
\text{Heterobeltiosis} = \frac{F_1 - \text{Better parent value}}{\text{Better parent value}} \times 100
\]

\[
\text{Standard heterosis} = \frac{F_1 - \text{Standard parent value}}{\text{Standard parent value}} \times 100
\]
F₁ PROGNEY OF DWARF x TALL CULTIVARS

Plate: 16 S.4864 (Cauvery x (CxR)) with medium bush

Plate: 16a Normal size robusta type fruits on medium internodes

F₁ PROGNEY OF TALL x DWARF CULTIVARS

Plate: 17 S.4850 (Sln.9 x Cauvery) with medium bush spread

Plate: 17a Normal size robusta type fruits on medium internodes
3.7. **Classification of F1 population**

3.7.1. **Vegetative Parameters**

Plant classification is the basis to postulate the heritable behaviors of the variable plant population generated through the inbred lines of diverse genetic back ground with the help of measurable parameters. In the present study, data on quantitative characters were recorded on 532 individual plants in F1 populations comprising 320 numbers in Dwarf x Tall and 212 numbers in Tall x Dwarf crosses. Tall x Tall progeny was not used for the classification due to negligible variations in the morphological characters of the F1 population. Based on the characteristic features of the parent cultivars, the entire F1 population was categorized into three types of plants namely; the plants with the phenotypic similarity to the female parent, the plants resembling the male parent in appearance and the plants with intermediate characters.

Thus, whole plant population was classified as ‘Cauvery’ type (dwarf type), Intermediate and Tall type. The plants with bush spread ranging from 120cm to 180cm was considered to be the homozygous for dwarf character and the population spreading the bush from 240cm to 300cm were accounted as homozygous for tall type. The intermediate types were placed under heterozygous category with class interval of 180-240cm. The actual number of plants under each class was presented in the form of percentage. The characters such as leaf length and breadth, leaf area, fruit yield and related parameters were classified as low, medium and high.

3.8. **Morphological proportions - the new criteria for detection of genetic inheritance in F1 progenies**

The present study was confined to nine F1 hybrids and their eight parents involved in crossing during 1996-97 and the progenies were established in the subsequent year as given in the following tables.
F₁ PROGNEY OF TALL x DWARF CULTIVARS

Plate: 18 S.4854 (S.881 x Cauvery) with medium bush
Plate: 18a Normal size fruits on medium internodes

F₁ PROGNEY OF TALL x DWARF CULTIVARS

Plate: 19 S.4860 (Slim.B x Cauvery) with medium bush
Plate: 19a Normal size fruits on medium internodes
Table: 3.6. List of the parents and their hybrids

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parents</th>
<th>Sl. No.</th>
<th>F1 Hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sln.9 (HDT x Tafarikela)</td>
<td>1</td>
<td>S.4842 (Cauvery x Sln.9)</td>
</tr>
<tr>
<td>2</td>
<td>Tafarikela (Ethiopian collection)</td>
<td>2</td>
<td>S.4850 (Sln.9 x Cauvery)</td>
</tr>
<tr>
<td>3</td>
<td>Cauvery (HDT x Catimor)</td>
<td>3</td>
<td>S. 4844 (Cauvery x S.881)</td>
</tr>
<tr>
<td>4</td>
<td>S.881 (Rume Sudan wild arabica)</td>
<td>4</td>
<td>S.4854 (S. 881 x Cauvery)</td>
</tr>
<tr>
<td>5</td>
<td>Sln.6 (Arabica x Robusta)</td>
<td>5</td>
<td>S.4876 (Sln. 6 x Cauvery)</td>
</tr>
<tr>
<td>6</td>
<td>Sln.5B (S.333 x Devamachy hybrid)</td>
<td>6</td>
<td>S.4847 (Cauvery x Devamachy)</td>
</tr>
<tr>
<td>7</td>
<td>Devamachy hybrid</td>
<td>7</td>
<td>S.4855 (Cauvery x Tafarikela)</td>
</tr>
<tr>
<td>8.</td>
<td>S.4179 (Catimor x Catuai)</td>
<td>8</td>
<td>S.4860 (Sln.5B x Cauvery)</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>9</td>
<td>S.4863 (Tafarikela x S.4179)</td>
</tr>
</tbody>
</table>

Data on morphological parameters were recorded during the year 2008-09 and statistically analyzed. The test of significance in the parents as well as F1 hybrids was worked out by taking measurements of morphological characters of 7 plants per replication for each genotype replicated 4 times.

The same data was used to study the characters inherited from parents to the offspring. The observations on bush spread, stem girth, primary’s thickness, internodal length of primaries, number of secondary shoot, leaf length and leaf breadth was taken into consideration. Ratios between the parameters within changeable characters namely; bush span, stem girth and primary thickness and within non-changeable characters such as, primary internodal length, leaf length, leaf breadth, fruit length and breadth were separately calculated for the parents as well as hybrids. The ratio differences of the parents and hybrids were compared to find out the inheritance of characters in F1 populations. Simultaneously, percent increase in the observed values of the morphological characters were worked out to compare the morphological proportions whether the increase or decrease in the ratio was due to addition or deletion of one character or both the characters.

3.9 A new model for quantification of parental traits transmission

There are two types of traits generally noticed in the plants, one is qualitative and the other quantitative. The genetic analysis of qualitative traits are carried out by Mendelian law of inheritance while, the analysis of quantitative traits is done by several statistical tools.
F₁ PROGNEY OF TALL x DWARF CULTIVARS

Plate: 20  S.4876 (Sln.6 x Cauvery) with medium bush spread

Plate: 20a Normal size fruits on medium internodes

F₁ PROGNEY OF TALL x DWARF CULTIVARS

Plate: 21  S.4863 (Taffarikela x S.4179) with medium bush

Plate: 21a Normal size fruits on medium internodes
designed to study the genetic variability, heritability, combining ability, genetic advance and genetic gain (Poehlman and Sleper, 1995). Apart from these, there are some other statistical methods such as, characters correlation, genetic penetrance, heterosis and response to selection are also applied for genetic analysis of the parents as well as progeny. Genetic variability and selection for important traits are vital components that every plant breeder needs its application to achieve better success in evolving cultivars of high yield potential and other desirable agronomic traits.

The quantitative traits association between genotype and phenotype is a very complex subject and not yet completely understood by the biological scientists. This provides information to the breeders to locate the genetic basis for emergence of several qualitative and quantitative phenotypic characters (Falconer and Mackay, 1996; Singh, 2000; Naraian, 1993).

Subsequently, the quantitative traits are governed by the polygenic action where, the expression of one character depends not only on one but on other several genetic factors (Chaudhary, 1982). In this context, studying the genetic behavior and correlation between the characters plays a crucial role in plant breeding program to find out the inter-relationship of one character with other and their magnitude of closeness (Ferrao et al., 2008). All the above techniques have different applications in genetic analysis of plant population but none of them are useful for quantification of parental traits and the degree of influence of each parent that are usually transmitted to the F1 offspring. Therefore, an effort was made to develop, a new formula to measure the percentage of genetic influence by each parent cultivar involved in crossing for the quantitative traits. The percent parental influence for quantitative traits transmission in F1 hybrids was determined with the help of new formula considering an example indicated in the following para with the help of figures.
During the hybridization process each parent contributes 50 percent of its genetic traits which forms 100 percent in the F₁ progeny. The dominant character that comes from one parent suppresses the character transmitted from other parent. Under this situation, the character suppressed is known as recessive character. Whereas, in the case of co dominance, both the parents exhibit equal effect on F₁ offspring that produces intermediate character (Atherly et al., 1999). In quantitative traits, the intermediate is measured in terms of mid parent value. Any deviation in the character from the mid parent value is an indication of parental influence combined with environmental impact. When the parents and the progeny are nurtured under the same environment, any deviation from the mid parent value can be considered to be due to parental influence. The deviation may be plus (+) or minus (-). For example, a dwarf variety with 180.00cm plant height when crossed with another variety of 250.00cm plant bush spread the F₁ hybrid had 185.00cm plant height. If it was the case of co dominance, the F₁ hybrid could have produced a bush spread of \((180.00 + 250.00)/2 = 215.00\) cm. But, the F₁ had 185cm bush spread which is an indication of dominance of dwarf variety. When a state of complete dominance is witnessed, the F₁ hybrid was expected to have a bush spread similar to its dwarf parent with 180cm. The difference between the dwarf parent and its F₁ hybrid (185.00-180.00=5.00cm) was most likely by some influence of tall variety. Further, the difference between mid parent value and the value of each parent is [\((215.00-180.00=35cm)\) and \((250.00-215.00=35.00cm)\)] 35.00cm. The difference in the bush span of 35.00cm is caused by 50 percent influence of each parent therefore, to cause 1.0cm difference it would influence \((50/35)=1.43\) percent. This value 1.43 can be used as a factor and be multiplied by the value of increase or decrease to find out the percent genetic contribution by the parent for a given trait. Hence, \(1.43\times5=7.15\) percent contribution was from tall parent and the balance 92.85 percent contribution from dwarf parent.
F₁ PROGNEY OF TALL X TALL CULTIVARS

Plate: 22 S.4856 (Sln. 9 x Sln.11) with tall type canopy
Plate: 22a Normal size fruits on long internodes

F₁ PROGNEY OF TALL x TALL CULTIVARS

Plate: 23 S.4857 (Sln. 5B x Sln.9) with tall type canopy
Plate: 23a Long internodes with normal size fruits
Though both the parents have contributed 50 percent equally in F₁, their influence for expression of the character varied based on their genetic dominance.

![Diagram showing genetic contribution from Cauvery and Sln.9 in F₁ hybrid mean.]

Method for measurement of parental quantitative traits transmission in F₁ progeny of Cauvery x Sln.9 cross

Method: I

In the situation of complete dominance, the F₁ hybrid could have the plant bush spread of similar to its dwarf parent (180cm.) The difference between the dwarf parent and its F₁ hybrid (185-180=5cm) was caused most likely due to some influence of tall variety. Further, the difference between mid parent value and the value of each parent is [(215-180=35cm) and (250-215=35cm)] 35cm. The difference in the bush spread of 35cm is caused by 50% influence of each parent combined with environmental involvement therefore, to cause 1.0cm difference it would influence (50/35)=1.43%. This value 1.43 can be used as a factor and be multiplied by the value of increase or decrease to find out the percent genetic contribution by the parent for a given trait. Hence, 1.43 x 5=7.15% contribution was from tall parent and the remaining was 100-7.15=92.85% contribution from dwarf parent. Though the both the parents have contributed 50%-50% in F₁ but their influence for expression of the
character varied depending on the genetic dominance which is an inbuilt genetic power of expression in the living beings.

A. % Parental contribution of Cauvery the dwarf female parent for bush spread

Example:

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Genotype</th>
<th>Bush spread (BS) in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female Parent - Cauvery (Ω$_F$) =</td>
<td>180.0</td>
</tr>
<tr>
<td>2</td>
<td>Male Parent- Sln.9 (Ω$_M$) =</td>
<td>250.0</td>
</tr>
<tr>
<td>3</td>
<td>Dwarf type progeny of Cauvery x Sln.9 (Ψ$_D$) =</td>
<td>185.0</td>
</tr>
<tr>
<td>4</td>
<td>Mean contribution of both parents assuming 50% from each (Ø) = ( \frac{Ω_F + Ω_M}{2} ) = ( \frac{180 + 250}{2} ) = 215.0cm</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Difference between mid parent value &amp; one parent ( Д = (Ø - Ω_F) ) = 215.0-180.0= 35.0cm</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Difference between progeny &amp; female parent ( θ_D = (Ψ_D - Ω_F) ) = 185.0-180.0= 5.0 cm</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Genetic contribution by the individual parents (C) = 50%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Factor ( ā = \frac{C}{Д} ) = 50/35 = 1.43</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>% effect of male parent Sln.9 ( E_M = ā \times θ_D ) = 1.43x5.0 = 7.15</td>
<td></td>
</tr>
</tbody>
</table>
| 10    | % effect of female parent ‘Cauvery’ \( E_F \) =\( 100- (ā \times θ_D ) = (100-(1.43x5.0) = 92.85 \)

Formula for effect of female parent \( E_M = \left( \frac{C}{\frac{Ω_F + Ω_M}{2} - Ω_F} \right) \times Ψ_D - Ω_F \)

Therefore, \( E_M = ā \times θ_D , \ E_F = 100- (ā \times θ_D ) \)

\( (E_M = \text{Effect of male parent}, \ E_F = \text{Effect of female parent}) \)

Based on the above formula the parental contribution for inherent quantitative traits in the F$_1$ progeny was worked.
F₁ PROGNEY OF TALL x TALL CULTIVARS

Plate: 24 S.4859 (Slm.5B x S.795) with tall type canopy

Plate: 24a Normal size fruits on long internodes

F₁ PROGENY OF TALL x TALL CULTIVARS

Plate: 25 S.4861 (Slm.6 x S.795) with tall type canopy

Plate: 25a Normal size fruits on long internodes
Method: II

Similarly, the effect of tall parent can also be worked out using the same formula. For example, when a tall plant with 250cm ($\Omega_M$) bush spread was crossed with a dwarf plant of 180cm $\Omega_F$ bush spread it bred the F$_1$ hybrid of tall types with 240cm ($\Psi_T$). In this case, the bush spread of 240cm in F$_1$ indicated the dominance of tall character.

\[
\text{Mid parent value} = \frac{\Omega_F + \Omega_M}{2} = \frac{180 + 250}{2} = 215.0 \text{cm}
\]

The distance between tall parent value and F$_1$ hybrid value \( \theta_T = (\Omega_M - \Psi_T) = 250 - 240 = 10\text{cm} \)

The distance between mid parent value and tall parent value \( D = (\Omega_M - \Theta) = 250 - 215 = 35\text{cm} \)

Factor (\( \bar{a} \)) = \( \frac{C}{D} = \frac{50}{35} = 1.43 \text{ percent} \)

The effect of dwarf parent \( E_F = (\bar{a} \times \theta_T) = 1.43 \times 5.00 = 7.15 \text{ percent} \)

The effect of tall parent \( E_M = (100 - E_F) = 100 - 14.3 = 92.85 \text{ percent} \)

Formula \( E_F = \left[ \frac{C}{(\Omega_F + \Omega_M)/2 - \Omega_M} \right] \times (\Omega_M - \Psi_T) \)

Therefore, \( E_F = \bar{a} \times \theta_T \) and \( E_M = 100 - (\bar{a} \times \theta_T) \)

Therefore, the dwarfing effect was 7.15 percent and tall effect was 92.85 percent when both the parents had equal contribution of 50 percent each for bush spread. Though, both the parents had equal contribution but the genetic influence of the parents for bush spread was not equal due to the differences in their gene strength which we call it genetic dominance. The term co dominance is used when both parents have equal genetic strength that reflects in the F1 progeny as intermediate character.

Limitations of the new formula:

1. This cannot produce accurate result when the parents and the progeny are grown under two different environmental conditions.

2. This can be used only for estimation of transmission of quantitative traits.
3. This can only be applicable for estimation of trait transmission in F1 progeny.

**F₁ PROGENY OF TALL x TALL CULTIVARS**

Plate: 26. S.4875 (Sln.6 x Sln.9) with tall type canopy

Plate: 26a. Normal size fruits on long internodes
The above study showed that the above formula developed for measurement of quantitative traits transmission can well be applied in the F1 plant population to understand the degree of individual parental influence for a given trait. This also facilitates identification of dominant traits in the progeny came either from male or female parent.

3.9. Genetic analysis of interspecific hybrids

There are various techniques that are effectively utilized to analyze the genetic constitution of an individual or population. Among these, Mendel’s Law of inheritance is well explained and accepted by the biological scientific community. Based on one central idea, it was understood that the blending of characteristics contributed by the two parents produce the offspring with intermediate types between the parents. However, the correct explanation came from the published work of Gregor Mendel in 1886 where, he proposed the concepts of hereditary units that are equally inherited from each parent and determined the observable phenotypes of the hybrids.

3.10. Genetic analysis of interspecific hybrids

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In the present study, the F1 hybrids produced by crossing genetically different coffee cultivars namely; ’Cauvery’ a tetraploid and CxR (C. congensis x C. canephora var. Robusta) a diploid species of genus Coffea were examined for the variability in morphological traits.
and dominant characters controlling the major phenotypes were identified for commercial exploitation. Further, the association of imperative traits and their segregation pattern in F₂ population were also studied taking into account monohybrid and dihybrid inheritance. The monohybrid and dihybrid segregation behaviors were tested by chi square (χ²) test.

The basis of genetic segregation was formulated by taking phenotypic characters into account such as bush spread, main stem girth, number of primary shoots, thickness of the primary shoots and number of internodes/primary as well as their internodal length. Beside this, some of the morphological characters like; leaf length, leaf breadth and leaf area were also used to find out the segregation behavior considering two pairs of allelic combinations in the inter-specific hybrid. The following table depicts the phenotypic classes of the progeny derived from the inter-specific crosses of Cauvery (tetraploid arabica) and CxR (diploid robusta var.) cultivars used for the present study.

**Table: 3.7. The criteria for classification of F₂ population of Cauvery x (CxR) cross**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phenotypic class</th>
<th>Characters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Plant type</td>
<td>Tall/Dwarf type</td>
</tr>
<tr>
<td>2.</td>
<td>Main stem girth</td>
<td>Small/Large</td>
</tr>
<tr>
<td>3.</td>
<td>Thickness of primary shoot</td>
<td>Thin/Thick</td>
</tr>
<tr>
<td>4.</td>
<td>Number of primary shoots/plant</td>
<td>Low/High</td>
</tr>
<tr>
<td>5.</td>
<td>Length of primary shoot</td>
<td>Short/Long</td>
</tr>
<tr>
<td>6.</td>
<td>Number of internodes/primary</td>
<td>Low/High</td>
</tr>
<tr>
<td>7.</td>
<td>Internodal length of primary</td>
<td>Short/Long</td>
</tr>
<tr>
<td>8.</td>
<td>Number of secondary branches</td>
<td>Low/High</td>
</tr>
<tr>
<td>9.</td>
<td>Leaf length</td>
<td>Short/Long</td>
</tr>
<tr>
<td>10.</td>
<td>Leaf breadth</td>
<td>Narrow/Broad</td>
</tr>
<tr>
<td>11.</td>
<td>Leaf area</td>
<td>Small/Large</td>
</tr>
</tbody>
</table>

### 3.11. Molecular genetic analysis of interspecific hybrids

Among various markers available for genetic analysis in plants, molecular markers are more efficient, precise and reliable for discriminating closely related species and cultivars.
and therefore, widely used in marker assisted breeding. Among the many types of molecular markers, sequence-related amplified polymorphism (SRAP) has been demonstrated to be a useful tool in genetic analysis of different plant species (Li and Quiros 2001; Ferriol et al., 2003; Esposito et al., 2007; Merotto et al., 2009).

SRAP is a PCR based marker system that preferentially targets coding sequences randomly distributed throughout genome (Li and Quiros, 2001). Forward and reverse primers used in SRAP preferentially amplify exonic and intronic regions of the genome respectively and uncover polymorphic sequences resulting from variations in the length of introns, promoters and spacers among different populations and genotypes. Zaefizadeh and Golieb (2009) reported that SRAP markers possess 4 multiloci and multi-allelic features, which make them potentially more efficient for genetic diversity analysis, gene mapping and fingerprinting of genotypes. Recently, SRAP markers were also used for identification of cultivars and hybrids (Hao et al., 2008; Xuan et al., 2008; Liu et al., 2007). SRAP is highly reproducible and comparatively less expensive than other types of markers (Cravero et al., 2007). However, the potential of SRAP marker has not yet been tested in coffee. In the present study, SRAP marker approach is employed in genetic analysis of six arabica coffee hybrids to ascertain its suitability and efficiency.

3.11.1. Plant materials used for molecular genetic analysis

An interspecific hybrid cross was undertaken involving tetraploid *C. arabica* c.v. Cauvery (2n=44) and triploid *C. canephora* c.v. CxR (3n=33). The resultant F₁ hybrids have distinct morphotypes one resembling the maternal parent Cauvery and the other largely similar to the paternal parent CxR with intermingling features of Cauvery. F₂ progeny was derived from the F₁ CxR type of plants (exhibiting morphological similarity with CxR parent plants). Based on their phenotypic features, F₂ plants were grouped into four different types as follows:
1. Cauvery type- with phenotypic appearance of arabica variety ‘Cauvery’

2. CxR robusta type- showing similarity with robusta plants of larger leaves and bush type character

3. Intermediate type- exhibiting admixture of arabica and robusta features

4. Off-types with abnormal leaf and fruits

Table: 3.8. Parents and hybrid combination analyzed by using SRAP marker.

<table>
<thead>
<tr>
<th>Parents</th>
<th>Hybrids of Cauvery x (CxR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cauvery/Catimor</td>
<td>a. Cauvery type</td>
</tr>
<tr>
<td>2. C x R Triploid form (3n=33)</td>
<td>b. CxR type</td>
</tr>
<tr>
<td>1. F₁ hybrids</td>
<td>a. Cauvery type</td>
</tr>
<tr>
<td>2. F₂ generation</td>
<td>b. Robusta type</td>
</tr>
<tr>
<td></td>
<td>c. Intermediate type</td>
</tr>
<tr>
<td></td>
<td>d. Off-type</td>
</tr>
</tbody>
</table>

Fresh young leaves were collected from both the parents, 10 individuals plants of two different types of F₁ (i.e. few plants of F₁ showing arabica (Cauvery) phenotype and the remaining plants exhibiting robusta (CxR) phenotypes and 10 individual plants belonging to four different types of F₂ progeny as described earlier. Isolation of DNA was carried out using young tender leaves from the plants categorized as above.

3.11.2. Methods of DNA extraction

Genomic DNA was isolated from fresh young leaves using a modified CTAB method as described earlier by Mishra et al., (2011a). About 200 mg of fresh leaf tissue was ground to fine powder in liquid nitrogen, transferred to a 30 ml tube containing 5 ml preheated extraction buffer (2 percent CTAB (w/v), 100 mM Tris-HCL (pH 8.0), 25 mM EDTA, 2M NaCl and 0.1 percent beta- mercaptoethanol). The tubes were incubated at 60 °C for one hour with occasional shaking. After incubation, the tubes were cooled to room temperature and centrifuged at 6000 rpm for 20 min. The supernatant was transferred into a new tube and extracted twice with chloroform-isoamyl alcohol (24:1). The supernatant was transferred to 2
ml tubes, precipitated with 0.7 volume of isopropanol at room temperature for 30 min., and then centrifuged at 8000 rpm for 20 min at 4°C. The pellet formed after centrifugation was washed with 75 percent (v/v) ethanol for 10 min and dissolved in 60 µl of Tris-EDTA (1-10 mM). The concentration of DNA was measured using 0.8 percent agarose gel stained with ethidium bromide as well as via a UV spectrophotometer at 260 nm. The ratio of the absorbance at 260 and 280 nm (A\textsubscript{260/280}) was used to assess the purity of DNA. The re-suspended DNA was then diluted in sterile distilled water to obtain 10 ng/µl concentrations for use in amplification reactions.

3.11.3. Methods of Amplification of SRAP markers

SRAP primers used in this study consist of 13 forward & 16 reverse primers of Li and Quiros (2001) and their sequences are presented in Table 2.

Primers were selected for further analysis based on their ability to detect clear and distinct polymorphic amplification products in various samples. Sixteen SRAP primer combinations that produced clearly readable and distinct polymorphic fragments in parents and hybrids were further selected for PCR amplification. Polymerase chain reaction was carried out in an Eppendorf master cycler (Eppendorf, Germany).

The SRAP analysis was conducted by adapting the procedure described by Li and Quiros (2001) with minor modifications as described earlier (Mishra \textit{et al.}, 2011a and b). The reaction mixture of 20 µl containing 1x reaction buffer (75mM Tris-HCl pH 8.8, 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.01 percent Tween 20), 30 ng template DNA, 200 µM dNTP mixture, 2.5 mM MgCl\textsubscript{2}, 3 µM each of forward and reverse primers, 1.0 U Taq DNA polymerase and sterile doubled-distilled water. The amplification conditions selected for SRAP included 4 min initial denaturation at 96 °C; 5 cycles consisting of 1 min denaturation at 94 °C, 1.15 min primer annealing at 35 °C; and 2 min extension at 72 °C, followed by 30 cycles consisting of
1 min denaturation at 94º C, 1.15 min primer annealing at 50º C and 2 min elongation at 72 ºC and a final extension of 15 min at 72 ºC.

Table: 3.9. Sequences of SRAP forward and reverse primer and primer combinations used in parents and hybrid analysis

<table>
<thead>
<tr>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
<th>Polymorphic primers combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me1TGAGTCCAAACCGGATA</td>
<td>Em2 GACTGCGTACGAATTTGC</td>
<td>Forward</td>
</tr>
<tr>
<td>Me2TGAGTCCAAACCGGAGC</td>
<td>Em3 GACTGCGTACGAATTTGAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>Me3TGAGTCCAAACCGGAAT</td>
<td>Em4 GACTGCGTACGAATTTGA</td>
<td>Me1, Em4/Em12</td>
</tr>
<tr>
<td>Me4TGAGTCCAAACCGGACC</td>
<td>Em5 GACTGCGTACGAATTTGA</td>
<td>Me2, Em4/Em6/Em12/Em14</td>
</tr>
<tr>
<td>Me6TGAGTCCAAACCGGACA</td>
<td>Em6 GACTGCGTACGAATTTGA</td>
<td>Me3, Em3/Em9/Em11</td>
</tr>
<tr>
<td>Me9TGAGTCCAAACCGGAGG</td>
<td>Em9 GACTGCGTACGAATTTGA</td>
<td>Me4, Em11/Em16</td>
</tr>
<tr>
<td>Me10TGAGTCCAAACCGGAAAA</td>
<td>Em10 GACTGCGTACGAATTTGA</td>
<td>Me6, Em5</td>
</tr>
<tr>
<td>Me11TGAGTCCAAACCGGAAC</td>
<td>Em11 GACTGCGTACGAATTTGA</td>
<td>Me9, Em10</td>
</tr>
<tr>
<td>ME12TGAGTCCAAACCGGAAGA</td>
<td>Em12 GACTGCGTACGAATTTGA</td>
<td>Me10, Em13</td>
</tr>
<tr>
<td>Em13 GACTGCGTACGAATTTGA</td>
<td>Me12, Em16</td>
<td></td>
</tr>
<tr>
<td>Em14 GACTGCGTACGAATTTGA</td>
<td>Em16</td>
<td></td>
</tr>
<tr>
<td>Em16 GACTGCGTACGAATTTGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR products obtained from SRAP analysis were analyzed via electrophoresis on 2.0% (w/w) agarose gels containing 0.5 µg ethidium bromide/ml in 1x TAE buffer as previously described by Mishra et al. (2011 a). The amplified bands were visualized and photographed using the UV-transilluminator (SYNGENE) and documented using the Gene Snap software program. All the three PCRs were repeated at least twice to confirm the reliability and repeatability of each PCR amplified band. The SRAP-amplified bands obtained with different primers were scored for presence (1) or absence (0) in data matrix form. Ambiguous bands that could not be easily distinguished were not scored the total number of bands, distribution of bands among the parents and hybrids, polymorphic bands, parental and hybrid specific bands and average number of bands per primer were manually calculated. The similarity of samples was calculated as follows: Similarity = $2N_{AB}/N_A+N_B$, $N_{AB}$ is the number of bands shared by individuals A & B, $N_A$ & $N_B$ are the number of bands in individuals A & B respectively (Chapco et al. 1992, Wild et al. 1992).

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