Materials and Methods

3.1 Materials:
On the basis of diversity in vegetative and reproductive characters the 15 indigenous germplasms viz., VB-67, V-8-Sheera, V-SWB-18, Seklar, V-JTS-8, V-SWB-32-10-1, Rama (collected from the germplasm bank of the Pulse and Oil Research Institute Berhampore of Murshidabad district) and the varieties collected from the districts of Burdwam, Hooghly, Howrah, South 24 Parganas, East Midnapore and West Midnapore of Sesamum indicum L. are used in this study. V-JTS-8, VB-67, V-SWB-32-10-1 are high yielding selection from 15 different cultivated germplasms and are recommended for cultivation in West Bengal. The remaining germplasms are not recommended for cultivation in this state.

Table 2.1: List of Sesamum species is used in this study.

<table>
<thead>
<tr>
<th>Germplasm No.</th>
<th>Sesamum Sp.</th>
<th>Place of Collection</th>
<th>Mean Latitude</th>
<th>Mean Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>V01</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V02</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V03</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V04</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V05</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V06</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V07</td>
<td>Sesamum indicum L.</td>
<td>Murshidabad</td>
<td>24.14° N</td>
<td>28.26° E</td>
</tr>
<tr>
<td>V08</td>
<td>Sesamum indicum L.</td>
<td>Burdwam</td>
<td>23.24° N</td>
<td>87.86° E</td>
</tr>
<tr>
<td>V09</td>
<td>Sesamum indicum L.</td>
<td>Hooghly</td>
<td>22.89° N</td>
<td>88.40° E</td>
</tr>
<tr>
<td>V10</td>
<td>Sesamum indicum L.</td>
<td>Howrah</td>
<td>22.59° N</td>
<td>88.31° E</td>
</tr>
<tr>
<td>V11</td>
<td>Sesamum indicum L.</td>
<td>South 24 Parganas</td>
<td>22.53° N</td>
<td>88.33° E</td>
</tr>
<tr>
<td>V12</td>
<td>Sesamum indicum L.</td>
<td>South 24 Parganas</td>
<td>22.53° N</td>
<td>88.33° E</td>
</tr>
<tr>
<td>V13</td>
<td>Sesamum indicum L.</td>
<td>East Midnapore</td>
<td>22.30° N</td>
<td>87.91° E</td>
</tr>
<tr>
<td>V14</td>
<td>Sesamum indicum L.</td>
<td>East Midnapore</td>
<td>22.30° N</td>
<td>87.91° E</td>
</tr>
<tr>
<td>V15</td>
<td>Sesamum indicum L.</td>
<td>West Midnapore</td>
<td>22.43° N</td>
<td>87.33° E</td>
</tr>
</tbody>
</table>
Fig. 1.1: Map of West Bengal and the colourful districts indicate the source of germplasms collection listed in Table- 2.1
3.2 Methods:

3.2.1 Growing the plants:
During the pre-kharif season, five 5 meter long rows with 50 cm spacing between the rows and plants of each of the 15 germplasms were sown in the experimental garden of Department of Botany, University of Kalyani, Kalyani, and West Bengal, India. The crop was raised under minimum management, without application of fertilizers and irrigation. Only one weeding was done at early stage. Five healthy plants were randomly chosen from the rows of each germplasms and labeled.

3.2.2 Location, Soil and Climate of the Experimental Garden:
Experiments was conducted in the medicinal and aromatic plant garden of Department of Botany, University of Kalyani, West Bengal, India, which is located at 22°57’N latitude, 88°22’E longitude with an average altitude of 9.75 m above mean see level. Information on soil of the garden is furnished below.

Table 2.2: Physico-Chemical properties of soil:

<table>
<thead>
<tr>
<th>Particular</th>
<th>Value</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Soil pH</td>
<td>6.2</td>
<td>Beckman’s pH meter, 1:2.5, soil : water Suspension (Jackson, 1967)</td>
</tr>
<tr>
<td>2. Organic carbon %</td>
<td>0.346</td>
<td>Walkley Black Method (1934)</td>
</tr>
<tr>
<td>3. Total nitrogen %</td>
<td>0.0421</td>
<td>Modified Kjeldahl's Method (Jackson, 1967)</td>
</tr>
<tr>
<td>4. Available Phosphorus (kg ha⁻¹)</td>
<td>23.69</td>
<td>Olsen's Method (Jackson, 1967)</td>
</tr>
<tr>
<td>5. Available Potassium (kg ha⁻¹)</td>
<td>86.48</td>
<td>Flame Photometric Method (Jackson, 1967)</td>
</tr>
</tbody>
</table>

Source: Meteorological Research Station, AICRP on Agro meteorology (ICAR), BCKV, Kalyani, Nadia, West Bengal, India.

Following meteorological information were collected from adjacent Meteorological Research Station, AICRP on Agro meteorology (ICAR), BCKV, Kalyani, Nadia, West Bengal, India.
during the growing period of the plant in the year 2011 and 2012 (during which the experimentation was carried out).

3.3 Morphological characterization of *Sesamum indicum* L.: Standard morphological parameters were taken into consideration for the present study. 15 germplasms of sesame were grown in the experimental plant garden and morphological data were taken. The following morphological characters were considered during study of 15 different germplasms of sesame:

**Plant height:** The height of the plants was recorded at the time of harvest from the soil surface in centimeters.

**Number of leaves/plant (Harvest maturity):** At maturity, the total number of leaves produced on each plant was counted.

**Number of primary branches/plant:** The no of primary branches was counted before harvest.

**No of capsule/plant:** The total number of capsules per plant was recorded.

**Number of seeds/capsule:** Number of seeds per capsule was counted.

**Number of loculi/capsule:** Number of loculi per capsule was recorded.

**Capsule Length:** At mature condition in harvest time the length of the capsule was measured in centimeters.

**Capsule width:** At mature condition in harvest time the width of the capsule was measured in centimeters.

**Number of capsule/axil:** Number of capsule per axil in each plant was recorded.

**Germination time:** Germination time or days was recorded from the date of first sowing of seeds in field.

**Days to the first flowering:** Number of days to flowering was calculated from the date of sowing to the date of first flowering.

**Days to the first capsule:** Number of days for capsuling was calculated from the date of sowing to the date of first capsuling.

**100 seeds weight:** Weight of 100 seeds was recorded in gram.

**Total lipid/oil obtained:** Total lipid or oil content was determined.

**Percentage of total lipid/oil (w/w):** Seed oil content was determined as percentage.
Fig. 1.2: Field showing sesame plants
3.4 Statistical analyses of morphological data:

Necessary statistical analyses were performed using the statistical software SPSS 7.5 (Copyright by SPSS Inc, USA, 1997, Base 7.5 Application Guide).

3.4.1 Analysis of Variance (ANOVA):

The ANOVA is a simple arithmetical process of sorting out the components of variation in a given data. According to Fisher, it is a tool by which the total variation may be split up into several assignable components. The variations of observations (data) with respect to any character (viz. Capsule length) as encountered in an experiment or within a population occur due to number of factors known as source of variation and the portions of variation caused by different sources are known as component of variation.

ANOVA for fifteen morphological characters of 15 germplasms of Sesame were carried out separately and experiments were laid out in a randomized block design (RBD) with five replications. The analysis was carried out using the above statistical software. The basic ANOVA table is given below.

Table 2.3: Basic Table for ANOVA (RCBD)

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f</th>
<th>SS</th>
<th>MSS</th>
<th>F value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>r-1</td>
<td>Repl SS</td>
<td>Repl MS</td>
<td>Repl MS/ MSE</td>
<td></td>
</tr>
<tr>
<td>Clone Type</td>
<td>c-1</td>
<td>Cl SS</td>
<td>CMS</td>
<td>Cl SS/ MSE</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>(r-1)(c-1)</td>
<td>MSE</td>
<td>MSE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>rc-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.f = degrees of freedom, SS = sum of squares, MSS = Mean sum of squares, F value = calculated F ratio, P = probability

3.4.2 Duncan’s test:

Randomized block design layout was followed to compare the mean of all morphological parameters under study of 15 different germplasms of Sesame. Standard error of means and critical difference at 1% and 5% level of significance (when necessary) were also calculated. After analyzing the experimental data using analysis of variance (ANOVA), significant differences among the means were tested by Duncan’s Multiple Range Test
(DMRT) using the statistical software SPSS 7.5 (Copyright by SPSS Inc, USA, 1997, Base 7.5 Application Guide).

3.4.3 Estimation of genetic parameters:

Estimation of all the genetic parameters like genotypic variance, phenotypic variance, genotypic and phenotypic coefficients of variation, heritability in broad sense ($h^2$), genetic advance (GA), genotypic and phenotypic correlation between pairs of traits were performed. The different terms, concepts and formulae are given in brief.

Estimates of coefficients of variation of different genetic parameters are obtained using the following formulae:

**Critical difference of the means**

$$\text{CD at 5\%} = SE_{d} \times t \text{ (at 5\% level or at 1\% level)}$$

**Genotypic variance**

$$\sigma^2_g = \frac{CMS - MSE}{r}$$

**Phenotypic variance**

$$\sigma^2_p = \sigma^2_g + \sigma^2_e$$

**Environmental variance**

$$\sigma^2_e = MSE$$

**Coefficients of variation (%)**

$$CV = \frac{\sqrt{MSE}}{r} \times 100$$

**Standard error of difference**

$$SE_{d} = \frac{\sqrt{2MSE}}{r}$$

**Genotypic coefficient of variation (GCV) (%)**

$$GCV = \frac{\sqrt{\text{Genotypic Variance}}}{\text{Grand mean}} \times 100$$

**Phenotypic coefficients of variation (PCV) (%)**

$$PCV = \frac{\sqrt{\text{Phenotypic Variance}}}{\text{Grand mean}} \times 100$$

**Heritability ($h^2$)**

$$h^2 = \frac{\text{Genotypic Variance}}{\text{Phenotypic Variance}}$$

**Genetic advance (GA)**

$$i \times h^2 \times \sqrt{\sigma^2_p}$$

(Where $i$ is the selection differential, $i= 2.06$ at 5\% selection intensity)

**Genotypic correlation ($r_g$)**

$$r_g = \frac{\text{cov}_g (X_1, X_2)}{\sqrt{\text{var}_g X_1 \cdot \text{var}_g X_2}}$$
Phenotypic correlation \( (r_p) = \frac{\text{cov}_p (X_1,X_2)}{\sqrt{\text{var}_p X_1 \cdot \text{var}_p X_2}} \)

Simple Correlation \( r(X_1X_2) = \frac{\text{cov}_p (X_1,X_2)}{\sqrt{v(X_1) \cdot v(X_2)}} \)

Where \( r (X_1 X_2) \) is the correlation between \( (X_1X_2) \)
\( \text{cov}_p (X_1,X_2) \) is the co-variance between \( X_1 \) and \( X_2 \)
\( v(X_1) \) is the variance of \( X_1 \)
\( v(X_2) \) is the variance of \( X_2 \)

3.4.4 Path analysis:
Path analysis helps to calculate the direct and indirect effects of different attributing causal characters on yield parameter (here, oil content), which are important agronomically to select suitable plant materials for generating plants with higher oil content. Genotypic and phenotypic correlation matrix was estimated using all agronomic characters including oil content. All these genotypic and phenotypic coefficients were tested by the t-test both at 1% and 5% level. These two matrices (Genotypic and Phenotypic) were again used for estimation of genotypic and phenotypic path. Here fourteen causal characters were utilized to see their causal effect (direct and indirect effect) on the percentage of total lipid/oil content.

3.4.5 Study of divergence Analysis:

3.4.5.1 Inter & Intra cluster distance study:
A measure for group distance based on multiple characters was given by Mahalanobis (1928) with \( X_1, X_2, X_3, \ldots, X_p \) as the multiple measurements available on each individual and \( d_1, d_2, \ldots, d_p \) as \( X_1^{-1} - X_1^{-2}, X_2^{-1} - X_2^{-2}, \ldots, X_p^{-1} - X_p^{-2} \) respectively, being the difference in the means of two populations, Mahalanobis’ \( D^2 \) statistic is defined as follow

\[ pD^2 = b_1d_1 + b_2d_2 + \ldots + b(pd) \]

Hare, the \( b_1 \ldots b_p \) values are to be estimated such that the ratio of variance between the populations to the variance within the populations is maximized. In terms of variance and covariance, the \( D^2 \) value is obtained as follows.

\[ pD^2 = W^{ij}(x_i^{-1} - x_i^{-2}) + (x_j^{-1} - x_j^{-2}) \ldots \ldots \ldots (1) \]

When, \( W^{ij} \) is the inverse of estimated variance covariance matrix.
The various steps involved in the estimations of $D^2$ values are given below:

i) Collection of data

ii) Test of significance

iii) Transformation of correlated variables.

iv) Computations of $D^2$ values.

v) Grouping of germplasms into various clusters (Tocher Method).

In the method the two, populations having smallest distance from each other are considered first to which a third populate having smallest average $D^2$ values from the first two populations is added. Then comes nearest fourth population and so eat goes on. At certain stage when it is felt that after adding a particular population, there is disrupt increase in the average $D^2$, this population is not added in that cluster. Similarly a second cluster is formed. Thus the process is continued till all the populations are included into one or the other cluster.

3.5 Analysis of Fatty acids composition of *Sesamum indicum* seeds by GLC:

Fifteen different Sesame seed samples were taken to analyze the fatty acids composition.

1. **Extraction of lipids:** Total lipids were extracted from seed samples by using hexane and methanol-chloroform (2:1, v/v) respectively for three and two times, each. The solvent extracts were pooled, dried over anhydrous sodium sulfate, filtered and the solvents were removed in a rotary vacuum evaporator, at room temperature, weighed and dissolved in distilled n-hexane and kept at $-20^\circ$C, till used.

2. **Preparation of methyl esters of fatty acids:** Total lipid samples were dissolved in anhydrous methanol containing concentrated Sulfuric acid (1.0%, v/v) and the mixture was refluxed for 2 hours. Methanol was evaporated to a small volume and cooled. Distilled water was added to the cooled mixture and the methyl esters of fatty acids were extracted 3 times with aliquots of diethyl ether. The ethereal extracts were pooled and dried over anhydrous sodium sulfate, filtered; vacuum dried, dissolved in n-hexane and kept in a freezer for future use.

3. **Purification of fatty acid methyl esters by thin layer chromatography (TLC):** Fatty acid methyl esters (FAMEs) were purified by TLC using a solvent system of n-hexane-diethyl ether (90:10, v/v). A standard fatty acid methyl ester was also run on the same plate in a separate lane. After development, the location of methyl ester bands were done by placing the TLC plate in iodine vapour chamber. The methyl ester bands corresponding to the standard were marked with...
a sharp needle and then scrapped off the plate. Methyl esters were recovered by extracting the bands by placing the scrapped out bands in a mini glass column and eluting with chloroform, the later was evaporated and the methyl esters were dissolved in n-hexane and kept in a freezer, till analyzed by GLC.

4. Gas-liquid chromatography (GLC): GLC of fatty acid methyl esters obtained by TLC, were done on a Chemito 1000 instrument by Chemito-Toshniwal, equipped with a flame ionization detector (FID). Quantifications were done by a computer using specific Clarity Lite software. GLC column used was a BPX-70 megabore capillary column of 30-mt long and 0.53 mm i.e. Oven temperature for fatty acid methyl ester was programmed from 150°C- 240°C @ 8°C/min. Initial and final temperatures were kept isothermal for 1 minutes and 10 minutes respectively. Injection port and detector temperatures were 250°C and 300°C respectively. Nitrogen gas was used as carrier, with a flow rate of 6.18ml/min.

Identification of fatty acids was done by comparing their retention times with those of standards, chromatographed under identical operational conditions of GLC. A secondary standard consisting of cod liver oil fatty acid methyl esters was also used to identify the component fatty acids in the sample, as suggested by Ackman3.
Sesame seeds

Ground with

(1) n-Hexane x3
(2) chloroform:methanol x2

Supernatants

Residue

Pooled

Pooled Sups

Vacuum dried and weighed

Total lipids (TL)

Refluxed with MeOH

Fatty acid methyl esters (FAME’s)

Purified by TLC

Purified FAME’s

GLC of purified FAMEs

Fig-1.3: Flow chart for the analysis of the fatty acids of sesame seeds
3.6 Genomic DNA extraction and purification

3.6.1 Stock Chemicals Required for DNA extraction:

A. 100 ml 1(M) Tris-HCl (p<sub>H</sub>=8.0):

This is an abbreviation of the organic compound Tris (hydroxymethyl) aminomethane, with the formula (HOCH<sub>2</sub>)<sub>3</sub>CNH<sub>2</sub>. The molecular weight of Tris salt is 121.14. For preparing 100ml of 1M Tris-HCl solution 12.114 g Tris (Promega, USA) was weighed and was dissolved in 80 ml double distilled water in a 250 ml conical flask. The p<sub>H</sub> of the solution was adjusted to 8.0 by drop wise addition of concentrated HCl (Merck). After p<sub>H</sub> adjusting, the solution was transferred to a measuring cylinder and the final volume was adjusted to 100ml with double distilled water. The 100 ml 1M Tris-HCl solution was sterilized by autoclaving at 15 psi for 15 min.

B. 50 ml 0.5 (M) EDTA (p<sub>H</sub> 8.0):

EDTA is an abbreviation for the chemical compound ethylene diaminetetra acetic acid [(HO<sub>2</sub>CCH<sub>2</sub>)<sub>2</sub> NCH<sub>2</sub>CH<sub>2</sub>N (CH<sub>2</sub>CO<sub>2</sub>H)<sub>2</sub>]. The molecular weight of EDTA is 292.25. For preparing 50 ml of 0.5 (M) EDTA solution (p<sub>H</sub> 8.0), 7.3063 g EDTA (Sisco Research Laboratories, India) was weighed and was dissolved in 35 ml of double distilled water in a 100 ml conical flask. The p<sub>H</sub> of the solution was adjusted to 8.0 by the addition of 5 (N) NaOH (Merck). After fixing the p<sub>H</sub>, the solution was transferred to a measuring cylinder and the volume was made up to 50 ml by the addition of double distilled water. The solution was then autoclaved at 15 p.s.i for 15 min and stored at 4° C.

C. Preparation of T<sub>10</sub>E<sub>1</sub> buffer:

Extracted genomic DNA was diluted and stored in high salt TE buffer. TE buffer was prepared with 10 mMTris-Cl (p<sub>H</sub> 8.0) and 1mM EDTA (p<sub>H</sub> 8.0) with 1M NaCl (Merck).

D. Preparation of TE Saturated Phenol:

Solidified distilled Phenol (MB grade) (Sisco Research Laboratories, SRL, India) was allowed to liquefy at 65° C water bath for 1h. 100 ml liquid phenol was measured in a measuring cylinder (wrapped with aluminium foil, to avoid light) with the help of a sterilized glass pipette, and was transferred to a sterilized amber bottle again wrapped with aluminium foil. 99 ml Tris-HCl (p<sub>H</sub> 8.0) was added (for saturating 100 ml phenol) and the bottle was stirred continuously with the help of a magnetic stirrer for 1h. The solution was then allowed to stand for separation of the two
layers. The upper Tris-Cl layer was removed carefully with a glass pipette and poured in a measuring cylinder. 50 ml double distilled water (i.e. half of the volume of phenol) was taken in a sterilized conical flask and few ml water was poured into the bottle containing phenol. The bottle was again stirred for 30 min and upper Tris-Cl layer was again transferred to the measuring cylinder and few ml double distilled water was poured into the bottle. This procedure was repeated to replace 50 ml double distilled water.

In another sterilized conical flask, 100 ml autoclaved double distilled water, 1 ml (M) Tris-HCl were taken and 200 μl 0.5 (M) EDTA was added to it and the solution was transferred to an amber bottle containing phenol. A pinch of 8-Hydroxyquinoline (Merck, India) was added. The bottle was stirrer and was stirred continuously overnight on a magnetic stirrer. Next day the Tris-EDTA saturated phenol was collected and stored in an amber bottle at 4°C.

**E. 5 (M) NaCl Solution:**

Molecular weight of NaCl is 58.44. 2.922 gmNaCl (Merck, India) was weighed and dissolved in 10 ml double distilled water to prepare 10 ml solution of 5 (M) NaCl stock.

### 3.6.2 Preparation of CTAB DNA extraction buffer:

CTAB (Cetyltrimethylammonium bromide) (Merck) extraction buffer was prepared with 100 mMTris-HCl (pH 8.0), 25 mM EDTA, 250 mMNaCl and CTAB (2%). 50 ml CTAB extraction buffer was prepared by mixing the stock the solution reagents in desired amounts in a sterilized conical flask.

<table>
<thead>
<tr>
<th>Amount taken from stock solution/weighed</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml 1(M) Tris-Cl (pH 8.0)</td>
<td>100 mMTris-Cl (pH 8.0)</td>
</tr>
<tr>
<td>2.5 ml 5 (M) NaCl</td>
<td>250 mMNaCl</td>
</tr>
<tr>
<td>2.5 ml 0.5 (M) EDTA (pH 8.0)</td>
<td>25mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>1 gm CTAB</td>
<td>CTAB (2%)</td>
</tr>
</tbody>
</table>

Note: During extraction, 0.2% 2-Mercaptoethanol (v/v) and 1.5% Polyvinyl pyrrolidone, PVP (w/v) were freshly added to the extraction buffer. All the reagents are procured from Merck.

The reagents as measured and mixed were transferred to a measuring cylinder, and the final volume was made to 50 ml with sterile double distilled water. Finally, the extraction buffer was stored into a sterile conical flask prior to use.
100 ml 3 (M) Sodium Acetate ($p^H$ 5.0):

The molecular weight of sodium acetate is 82.02. For preparing 100ml of 3 (M) sodium acetate solution ($p^H$ 5.0), 21.61 g sodium acetate (anhydrous) (Sisco Research Laboratories, India) was weighed and dissolved in 80 ml of double distilled water in a conical flask. The $p^H$ of the solution was adjusted to 5.0 by addition of glacial acetic acid. After adjusting the $p^H$, the solution was transferred to a measuring cylinder and the volume was made up to 100 ml by addition of double distilled water. The solution was then transferred to conical flask, autoclaved at 15 lb pressure for 15 min.

3.6.3 Sterilization of Equipments:

Mortar, pestle, measuring cylinder (Borosil, India), glass pipette (Borosil, India), Bottle (amber coloured), conical flasks (Borosil) were washed properly with tissue culture-grade detergent (Teepol purchased from local market), rinsed with distilled water and dried in hot air oven. All the equipments, eppendorf tubes (1.5 ml, Tarson), Pipette tips (1 ml, 200 μl and 10 μl, Tarson) and capillary tubes were sterilized by autoclaving at 15lb pressure for 15 min.

3.6.4 Modified CTAB method:

**Equipment:**
1. Centrifuge
2. Water bath
3. 2 ml micro centrifuge Eppendorf tube
4. Mortar and pestle (Pre-Chilled)
5. UV- 1601 spectrophotometer (Shimadzu, Japan)
6. 10ml Polypropylene Centrifuge tube
7. Incubator (37°C)

**Reagents and Chemicals required:**
1. Liquid nitrogen
2. 2% w/v CTAB (Hexadecyltrimethylammonium bromide)
3. Tris-HCl $p^H$ 8.0 (1.0M)
4. EDTA $p^H$ 8.0 (100ml) (Ethylenediaminetetra acetate acid)
5. 1.5 M NaCl
6. 0.2% β-mercapto ethanol (v/v)
7. 1% PVP (Polyvinyl pyrrolidone)
8. 3M Sodium acetate- $P^H = 5.0$
9. 70% ethanol $-20^\circ C$
10. Absolute isopropanol $-20^\circ C$
11. Chloroform: Isoamyl alcohol (24:1 v/v)
12. RNase A (10mg/ml) in TE ($P^H = 8$)
13. Phenol (Equilibrated)

**Extraction buffer:**
1. 100 mMTris-Cl ($P^H 8.0$)
2. 20 mM EDTA
3. 1.5 M NaCl
4. 2% CTAB
5. 0.2% $\beta$-mercaptoethanol(v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use)

**TE buffer:** 10 mMTris-Cl ($P^H 8.0$) and 1 mM EDTA ($P^H 8.0$)

**3.6.5 Plant samples for DNA isolation:**

Genomic DNA was extracted from seeds of *Sesamum* species based on Cetyltrimethyl ammonium bromide (CTAB) procedure (Murry & Thompson 1980) with minor modification. Fresh seeds (1g) were collected, treated with teepol (Extran) for 5 minutes and rinsed with sterile double distilled water. Surface moisture was soaked with tissue. seed were grinded in liquid nitrogen.

**3.6.6 DNA Extraction Protocol:**

**A. Extraction Step:**
1. Grind the 1 gm fresh seeds in liquid nitrogen with mortar and pestle.
2. Transfer the ground power or paste to clean autoclaved 10 ml polypropylene tube. Add 5 ml extraction buffer. Mix by inversion to slurry.
3. Incubate homogenate at 65$^\circ C$ for 1 hour in a water bath with intermittent shaking.
4. Add an equal volume of Chloroform: isoamyl alcohol (24:1) and mix by inversion for about 1 minute.
5. Centrifuge at 15000 rpm for 10 minutes at 4°C.
6. Transfer the aqueous phase to a fresh polypropylene tube and add 2/3 volume of ice cold Isopropanol and mix by quick gentle inversion for about 2 minutes.
7. Precipitate the DNA by incubating either 30 minutes or overnight (12h) at -20°C.
8. Centrifuge at 6000 rpm for 10 minutes at 4°C.
9. Discard the supernatant gently. Wash the pellet with 1000 µl 70% chilled ethanol (v/v).
10. Centrifuge at 10000 rpm for 5 minutes at 4°C. Repeat steps 3-4 times.
11. Air dries the pellet for about 30 minutes at room temperature. Resuspend the pellet in 1 ml of sterile milliQ water or TE buffer.

B. Purification step:
1. Add 10 µl of 10 mg/ml DNase free RNase A. Incubate at 37°C for 1 (h).
2. Add equal volume of Phenol: Chloroform (1:1) and mix by gentle inversion for 2 minutes.
3. Centrifuge at 11000 rpm for 10 minutes at 4°C.
4. Transfer the upper phase to a sterile micro centrifuge tube and repeat the extraction with Chloroform: isoamyl alcohol (24:1) twice.
5. Centrifuge at 5000 rpm for 5 minutes at 4°C.
6. To the supernatant, add 0.1 vol. of 3 (M) Sodium acetate and 2/3 vol. of isopropanol. Mix by gentle inversion and incubate at -20°C for 15 minutes precipitate DNA.
7. By Centrifuging at 10000 rpm for 5 minutes at 4°C and wash with pellet with 1ml of 70% chilled ethanol 3 times.
8. Air dry and dissolve pellet is 100 µl sterile milli-Q water or TE buffer.

3.6.7 Characterization of Isolated DNA:
Quantification: Isolated DNA was quantified using spectrophotometer (Cecil, Germany). 998 µl of TE buffer was taken in a quartz cuvette and 2 µl of extracted genomic DNA was added to it. The optical density (absorbance A) was taken at 260 nm (A260) and 280 nm (A280). The amount of the DNA present in the solution was calculated from absorption at 260 nm (A260) and the purity of DNA was calculated by A260/A280 ratio. For an ideal DNA preparation the A260/A280 ratio should be ≥ 1.8 (Sambrook et al. 2001)
Formula for calculating the DNA concentration:

\[
\text{DNA concentration} = \text{Spectral reading (A}_{260} \times 50\mu\text{g/ml} \times \text{dilution factor}
\]

For double stranded genomic DNA, 1 O.D. corresponds to 50 μg/ml of DNA (Sambrook et al. 2001).

\[
\text{Dilution factor} = \frac{998\mu\text{L TE buffer} + 2\mu\text{L DNA}}{2\mu\text{L}} = 500
\]

3.6.8 Qualification of Isolated DNA:

The quality of the extracted DNA was tested by running the DNA in 0.8% agarose gel.

Solution for gel electrophoresis:

- **Running buffer (1X TAE):**

Stock solution of 50X TAE buffer containing Tris (2M), EDTA (50mM) and glacial acetic acid (5.71%, Merck, India) was prepared. For 100 ml of 50X stock solution preparation 24.22g Tris (Promega), 1.85g EDTA (SRL, India) and 5.71ml glacial acetic acid were dissolved in 80ml of double distilled water. The pH of the solution was adjusted to 8.0 with drop wise addition of concentrated HCl, and final volume was made up to 100 ml with double distilled water. The 50X TAE buffer was autoclaved at 15 p.s.i pressure for 15 minutes and stored at room temperature. During gel electrophoresis, 50X TAE buffer was diluted with sterile double distilled water to obtain 1X TAE buffer (working concentration) before running the gel.

- **Gel loading Buffer (6X):**

To prepare 25 ml of gel loading buffer (6X) 30 mg Bromophenol blue dye (Sigma) was mixed with 9.36 ml of 80 % glycerol (Merck), 30 mg xylene cyanol (Merck) and 300 μl EDTA (0.5 M). The final volume was made with 15.34 ml sterile double distilled water and the solution was vortexed briefly and stored at room temperature. During loading of DNA of gel loading buffer was diluted to 1X with addition of 1X TAE.

- **Gel staining dye:**

10 mg Ethyldium bromide (Genei, Bangalore, India) was weighed in an eppendorf tube and dissolved in 1ml sterile double distilled water. The solution was mixed and vortexed thoroughly. The solution was stored at room temperature, and used for staining the DNA gel at a working concentration of 0.5μg/ml (Sambrook et al. 2001).
• **Gel casting:**

The isolated DNA was analysed for its quality and integrity by running in 1.0% agarose gel in 1X TAE buffer system. For gel casting, 1.0 g of Agarose (SRL, India) was weighed and mixed with 1X TAE buffer (2ml 50X TAE and 98ml distilled water). The agarose was solution warmed in a heater and was allowed to melt resulting in a clear solution. Meanwhile the gel casting tray was sealed with steel grip tape and properly placed in a horizontal gel casting table. When the agarose solution cooled down to about 60°C, 5 µl Ethidium bromide (Genei, Bangalore, India) was added, was poured carefully into the sealed gel tray, immediately placing the comb, avoiding any air bubble. The gel was allowed to solidify for 45 minutes. After solidification, the comb and seal tape was carefully removed, and the gel was placed in the gel trough with proper (Orientation with respect to cathode and anode), and immersed with sufficient 1X TAE buffer.

• **Gel electrophoresis:**

For electrophoresis of DNA sample 5 µl DNA was mixed with 5 µl 1× TAE and 2 µl gel loading dye on a parafilm paper and loaded onto each well. The gel was allowed to run at a constant voltage of 60 V (5V/cm distance between electrodes) for 1.20 hours till the tracking dye reaches middle of the gel. The gel was examined under ultraviolet transilluminator and photographed using Gel Doc system 1000 (Bio Rad).

### 3.6.9 PCR amplification of *Sesamum* DNA using RAPD

A set of 25 randomly selected oligonucleotide primers were used for RAPD assay of 15 germplasms of *Sesamum* (Fig.2.34). Out of 10 RAPD primers produced consistent and reproducible patterns of amplified products in all the germplasms. The details on the sequence of the primers are given in Table- 2.5.

#### 3.6.9.1 Primer Hydration and Quality check:

All the primers (oligos) for RAPD were procured from Genei, Bengalore, India and were supplied as lyophilized powder. The hydration was performed by adding 100 µl sterile PCR grade water (Genei, Bengalore, India), and incubation the vial at 65°C for 10 minutes. After tapping and vortexing for a brief period, the vials were stored at -20°C.
3.6.9.2 Formula to calculate amount of oligos supplied:

Warburg and Christian calculated a way to relate absorbance to specific factors and equations to give a concentration in µg/ml. Warburg and Christian determined these equations and factors based on the relationship between the molar absorptivity values of yeast RNA and protein called enolase, determined at A_{260} and A_{280} nm with background correction at A_{320} nm (Warburg & Christian 1942; Sambrook et al. 2001).

The average mass of nucleotide base in DNA is 33 g/mol. A pure, single standard DNA (oligo) solution that is 33 µg/ml will have an absorbance of 1.0 at 260 nm. Thus A_{260} =1.0 =33 µg/ml

Therefore, from 100 µl suspended aligos, 2 µl was taken into 1 ml double distilled water and absorbance was measured at 260 nm.

$$2 \mu l \text{ oligo} = \text{“x” O.D}$$

$$\text{Total amount of oligos in } 100 \mu l \text{ of volume} = \frac{33 \mu g \times “x” \times 100}{2\mu l}$$

3.6.9.3 PCR Amplification:

PCR reaction for RAPD was carried out using amplification reagents (Genei, Bangalore, India) listed below. All the PCR amplification reactions were performed in Gene Amp 2400 PCR system (Perkin Elmer).

3.6.9.4 Chemical required for PCR reactions:

- Assay buffer (10X): Assay buffer containing 100 mMTris-HCl (pH 9.0), 500 mM KCl and 0.01% gelatin (w/v), without MgCl$_2$ was diluted to 1X during PCR mix preparation (Genei, Bangalore, India).

- dNTP Mix: Each dNTP was supplied as 10mM stock. These were diluted to 2mM as working standard with PCR grade water (Genei, Bangalore, India). 20 µl of each dNTP was added from 10 mM stock, to 20 µl sterile PCR grade water in a sterile vial, to prepare dNTP mix of 100 µl with 2 mM of each dNTP (Genei, Bangalore, India).

- MgCl$_2$: 25 mM MgCl$_2$ solution was supplied (Genei, Bangalore, India).

- Taq DNA polymerase: Taq DNA polymerase of 3U/µl strength without gelatin was used in all PCR reactions (Genei, Bangalore, India).
3.6.9.5 RAPD-PCR with Sesamum genomic DNA:

RAPD Amplification was performed with extracted and purified genomic DNA from 15 germplasms of *Sesamum* using 10 RAPD primers sets (Genei, Bangalore, India) (Table 2.5). Each RAPD PCR mixture (50 μl) contained 1 μl genomic DNA (template), 2 μl of 10 mM dNTPs mixture, 5 μl 10X Taq polymerase buffer, 1μl MgCl2 (final concentration of 1.0 mM MgCl2), 3U of Taq DNA polymerase enzyme and 400ng RAPD primers. The final volume of 50 μl was made up with PCR grade water (Genie, Bangalore, India). Amplification reactions were performed with an initial denaturation at 94°C for 5 minutes, followed by 45 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 38°C, 2 minutes extension at 72°C with a final extension of 72°C for 5 minutes using thermal cycles (Perkin Elmer gene Amp 2400 PCR system) brought down to 4°C.

Table 2.5: Detail of the RAPD primers used for RAPD analysis of *Sesamum indicum* germplasms:

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Code</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TD1</td>
<td>TTTGGCGGCCCT</td>
</tr>
<tr>
<td>2</td>
<td>TD2</td>
<td>ACTCTGCGCAC</td>
</tr>
<tr>
<td>3</td>
<td>TD3</td>
<td>GAGGTTCCACA</td>
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<td>TGCCGAGCTG</td>
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<td>5</td>
<td>TD5</td>
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<td>TD6</td>
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<td>7</td>
<td>TD7</td>
<td>AGGTGACCGT</td>
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<td>AGGCGCGGAAC</td>
</tr>
<tr>
<td>10</td>
<td>TD10</td>
<td>AGGTCTTGGG</td>
</tr>
</tbody>
</table>

3.6.9.6 Agarose gel electrophoresis of PCR Products:

The PCR amplified products obtained from RAPD reactions with genomic DNA of *Sesamum* were visualized by running the PCR products in 1.75% agarose gel.

3.6.9.6.1 Gel Casting:

The PCR products were resolved on agarose gel (1.75%) with 1X TAE buffer system. 1.5g agarose (SRL, India) was weighed and 100 ml 1X TAE buffer (2 ml 50X TAE and 98 ml
distilled water) was added to it. The gel was according the gel casting procedure as mentioned earlier in this chapter.

3.6.9.6.2 Gel loading and Gel Staining:

The solidified agarose gel containing 0.5 μg/ml ethidium bromide (added to melted agarose solution before pouring) was placed within the gel trough immersed with sufficient 1X TAE buffer. 10 μl PCR products with 2 μl gel loading dye were added to all the wells. A low range DNA ruler (100-3000bp) (Genei, Bangalore, India) was used as known molecular weight marker. 1 μl marker DNA mixed with 2 μl gel loading dye (supplied along with marker) was loaded separately. Electrophoresis was done at a constant voltage of 70V for 1.30 hours until the tracking dye reaches just below the middle of gel. The gel was observed in a transilluminator and photographed in Gel Doc System (Biorad).

3.6.10 Data analysis:

Since the RAPD markers are dominant, genetic data analysis was carried out on the assumption that each band represented the phenotype at a single biallelic locus. Amplification with each arbitrary primer was repeated three times and consistent bands were selected for data generation. The presence and absence of bands were scored as 1 or 0, respectively. Faint bands were not recorded for analysis. By comparing the banding patterns of genotypes (germplasms) for a specific primer, genotype-specific bands were identified. The binary data so generated was used to estimate the levels of polymorphism by dividing the polymorphic bands by the total number of scored bands.

To analyze the suitability of RAPD markers to evaluate genetic profiles of Sesamum, the performance of the markers was measured using three parameters: (i) polymorphic information content (PIC), (ii) marker index (MI) and (iii) resolving power (RP).

The PIC value for each locus was calculated as proposed by Roldan-Ruiz et al. (2000);

\[ \text{PIC}_i = 2f_i (1-f_i) \]

Where, PICi is the polymorphic information content of the locus i, fi is the frequency of the amplified fragments (band present) and 1 - fi is the frequency of non-amplified fragments (band absent). The frequency was calculated as the ratio between the number of amplified bands at
each locus and the total number of accessions (excluding missing data). The PIC of each primer was calculated using the average PIC value from all loci of each primer.

The marker index (MI) was calculated as described by Varshney et al. (2007);

$$MI = EMR \times PIC;$$

where, EMR (effective multiplex ratio) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay.

The resolving power (RP) of each primer was calculated according to Prevost & Wilkinson (1999);

$$RP = \sum I_b;$$

where $I_b$ represents the informative fragments. The $I_b$ can be represented on a scale of 0-1 by the following formula:

$$I_b = 1 - [2 \times (0.5 - p)];$$

where $p$ is the proportion of accessions containing the band.

The genotype and allelic frequency data were used to compute the genetic diversity indices, i.e.,

(i) percentage of polymorphic loci [$P\% = (\text{polymorphic loci/total loci}) \times 100$],
(ii) observed (na) and effective number of alleles (ne), where ne in the number of equally frequent alleles that would be necessary to achieve the same the degree of genetic diversity produced by Na
(iii) Shannon’s Information Index (I) (Shannon & Weaver, 1949) calculated from

$$I = (\sum \pi_i \log \pi_i)$$

where $\pi_i$ is the frequency of RAPD fragments amplified in the accessions and L is the total number of fragments and (iv) Nei’s genetic diversity (h) (Nei 1973) derived from

$$h = 1 - \sum \pi_i^2$$

where $\pi_i$ is the frequency of the $i^{th}$ allele at the locus, were calculated with the aid of the POPGENE program version 1.31 (Yeh et al. 1999). For each locus, the Nei’s index produces values between 0 and 0.5, while the Shannon index varies from 0 to 0.73 according to a natural log scale (Lowe et al. 2004).
The binary data matrix was used to calculate Jaccard’s similarity coefficient between pairs of accessions using the Simqual module of NTsys-PC (Numerical Taxonomy System version 2.1) (Rohlf 1993). These distance coefficients were used to construct dendrogram using the Unweighted Pair Grouped Method Arithmetic Average (UPGMA) employing the Sequential Agglomerative Hierarchical and Nested (SAHN) algorithm for determining the genetic diversity and relationships among the accessions. In order to highlight the resolving power of the ordination, Principle coordinate analysis (PCA) was performed using the EIGEN and PROJ modules of NTSYS Pc.