MATERIALS AND METHODS

A. Morphological study

Sixty cultivars of *Hibiscus rosa-sinensis* L. were collected from different households in the urban areas of Thiruvananthapuram and Kollam. Authentic cultivar names are not available except for a very few, and hence the cultivars possessing features distinct from one another were assigned accession numbers from C-1 to C-60. Morphological characters were recorded from fresh plants. Herbarium specimens of the taxa studied are maintained at the Department of Botany, S.N. College, Kollam.

(a) Foliar and floral

A detailed morphological study was conducted collecting data from foliar and floral parts. Morphological characters were studied under two heads-quantitative and qualitative characters. The study includes eighteen quantitative and sixteen qualitative characters. For each quantitative character, observations from five plants per cultivar were taken, and the average was used for the analysis. The qualitative foliar characters were studied following the classification of leaf characters by Hickey (1973), and those concerning leaf venation are

1. Simple craspedodromous – all the secondary veins terminate at the margin.

ii. Mixed craspedodromous - a mixture of simple and semi craspedodromous types, with only some secondary veins terminating at the margin while the remaining secondaries branch near the margin and only one branch terminates while the other joins the super adjacent secondary.

iii. Brochidodromous – secondaries joined together forming arches just below the margin.

iv. Eucamptodromous– secondaries upturned and gradually diminishing towards the margin.

v. Reticulodromous – secondaries losing their identity towards the margin by repeated branching into a vein reticulum.

vi. Cladodromous – secondaries freely ramified towards the margin.

vii. Actinodromous – three or more primary veins diverging radially from a single point.

viii. Palinactinodromous– primaries having one or more subsidiary points of radiation above the lowest point.

The colour of the lamina was determined by comparing with the colour chart by Wilson (1938; 1941).

Details of the characters studied and their character states, codes and abbreviations are provided in Table 1. The quantitative characters were divided into three classes based on the property of normal distribution, 95% confidence intervals being used for the purpose as below

Low = below mean – 2 SD ($<2\sigma$)

Medium = mean ± 2SD ($\pm 2\sigma$)

High = above mean + 2 SD ($>2\sigma$)

Table 1: List of morphological characters studied and their respective character states, abbreviations and codes used in the cultivars of *Hibiscus rosa- sinensis* L.

Sl. No.	Characters	Character states	Abbreviations	Code
1	Petiole Length (PtL)	Low	PtLL	1
		Medium	PtLM	2
		High	PtLH	3
2.	Lamina Length (LaL)	Low	LaLL	1
		Medium	LaLM	2
		High	LaLH	3
3.	Lamina Breadth (LaB)	Low	LaBL	1
		Medium	LaBM	2
		High	LaBH	3
4.	Lamina Area (LaA)	Low	LaAL	1
		Medium	LaAM	2
		High	LaAH	3
5.	LaminaPerimeter (LaP)	Low	LaPL	1
		Medium	LaPM	2
		High	LaPH	3
6.	Stipule Length (StL)	Low	StLL	1
		Medium	StLM	2
		High	StLH	3
7.	Pedicel Length (PedL)	Low	PedLL	1
		Medium	PedLM	2
		High	PedLH	3
8.	Epicalyx Segment Number (EpSN)	Low	EpSNL	1
		Medium	EpSNM	2
		High	EpSNH	3
9.	Epicalyx Segment Length (EpSL)	Low	EpSLL	1
		Medium	EpSLM	2
		High	EpSLH	3

Contd...

10	Epicalyx Segment Breadth (EpSB)	Low	EpSBL	1
		Medium	EpSBM	2
		High	EpSBH	3
11	Calyx Length (CaL)	Low	CaLL	1
		Medium	CaLM	2
		High	CaLH	3
12	Petal Length (PetL)	Low	PetLL	1
		Medium	PetLM	2
		High	PetLH	3
13.	Petal Breadth (PetB)	Low	PetBL	1
		Medium	PetBM	2
		High	PetBH	3
14.	Staminal Column Length (SCL)	Low	SCLL	1
		Medium	SCLM	2
		High	SCLH	3
15.	Stamen Number (SN)	Low	SNL	1
		Medium	SNM	2
		High	SNH	3
16.	Filament Length (FiL)	Low	FiLL	1
		Medium	FiLM	2
		High	FiLH	3
17.	Style Length (StyL)	Low	StyLL	1
		Medium	StyLM	2
		High	StyLH	3
18.	Stigma Number (StiN)	Low	StiNL	1
		Medium	StiNM	2
		High	StiNH	3
19	Flower Type (FlT)	Single	SF	1
		Double	DF	2

Rou

Cou

Contd...

20	Leaf Colour (LeC)	Parsley Green	PaGL	1
		Spinach Green	SpGL	2
		Scheeles Green	ScGL	3
		Willow Green	WiGL	4
		Fern green	FeGL	5
		Variegated	VarL	6
21.	Leaf Shape (LeS)	Wide oblong	WObL	1
		Very wide oblong	VWObL	2
		Elliptic	ElL	
		Wide elliptic	WElL	4
		Suborbiculate	SOrbL	5
		Orbiculate	OrbL	6
		Narrow ovate	NOvL	7
		Ovate	OvL	8
		Wide ovate	WOvL	9
		Very wide ovate	VWOvL	10
		Lobed	LobL	11
22	Leaf Texture (LeT)	Chartaceous	ChaLT	1
		Coriaceous	CorLT	2
23	Leaf Margin (LeM)	Entire	EnLM	0
		Serrate	SeLM	1
		Lobed	LobLM	2
24	Degree of serration (DS)	Absent	DSA	0
		Low	DSL	1
		Medium	DSM	2
		High	DSH	3
25	Leaf Apex (LeA)	Acute	AcLA	1
		Obtuse	ObLA	2
		Rounded	RoLA	3
		Acuminate	AmLA	4
26	Leaf Base (LeB)	Acute	AcLB	1
		Obtuse	ObLB	2
		Rounded	RoLB	3
		Cordate	CoLB	4

Contd...

27	Leaf venation (LeV)	Simple craspedodromous	SiCLV	1
		Mixed craspedodromous	MiCLV	2
		Brochidodromous	BrLV	3
		Eucamptodromous	EuLV	4
		Reticulodromous	RetLV	5
		Cladodromous	ClLV	6
		Actinodromous	ActLV	7
		Palinactinodromous	PalLV	8
28.	Petal Colour (PetC)	White	WhPC	1
		Cream	CrPC	2
		Pink	PiPC	3
		Mustard	MuPC	4
		Yellow	YePC	5
		Saffron	SafPC	6
		Orange	OrPC	7
		Red	RePC	8
		Magenta	MagPC	9
		Mixed	MixPC	10
29	PetalBaseColour (PetBC)	White	WhPBC	1
		Pink	PiPBC	2
		Yellow	YePBC	3
		Light Red	LRePBC	4
		Red	RePBC	5
		Crimson	CriPBC	6
		Magenta	MagPBC	7
		Maroon	MarPBC	8
30	Staminal Column Colour (SCC)	Absent	ASCC	0
		White	WhSCC	1
		Cream	CrSCC	2
		Pink	PiSCC	3
		Yellow	YeSCC	4
		Saffron	SafSCC	5

Contd...

		Red	ReSCC	6
		Magenta	MagSCC	7
		Maroon	MarStiC	7
31	Anther Colour (AnC)	Absent	AAnC	0
		Cream	CrAnC	1
		Yellow	YeAnC	2
		Bright Yellow	BYeAnC	3
		Golden Yellow	GYeAnC	4
		Saffron	SafAnC	5
		Reddish Yellow	ReYeAnC	6
32	Filament Colour (FiC)	Absent	AFiC	0
		White	WhFiC	1
		Cream	CrFiC	2
		Pink	PiFiC	3
		Yellow	YeFiC	4
		Saffron	SafFiC	5
		Red	ReFiC	6
		Magenta	MagFiC	7
33	Stigma Colour (StiC)	Absent	AStiC	0
		Cream	CrStiC	1
		Yellow	YeStiC	2
		Orange	OrStiC	3
		Orange Red	OrReStiC	4
		Deep red	DReStiC	5
		Saffron	SafStiC	6
		Maroon	MarStiC	7
34	Fertility / Sterility (Fer/Ste)	Completely sterile	Ste	0
		Male Sterile	Ste M	1
		Female sterile	Ste F	2
		Fertile	Fer	3

The morphological data assembled have been subjected to statistical analysis as under;

1. Variability

Variability of all the quantitative characters studied was estimated by comparing the mean, range, standard deviation, variance, standard error, coefficient of variation and critical difference using appropriate formulae (Panse and Sukhatma, 1985).

2. Analysis of variance

ANOVA was used to determine whether mean differences exist between cultivars for each morphological character. The F-ratio was calculated using the formula

$$F = \frac{\text{Variance between sample means}}{\text{Variance expected from sampling error}}$$

The variance ratio was tested by Snedecor's F-test for (n-1) degrees of freedom at 5% and 1% levels of significance (Snedecor and Cochran, 1980).

3. Heritability

This is the fraction of phenotypic variance attributable to genetic difference among cultivars, and is defined in the broad sense as the ratio of the genotypic variance to the phenotypic variance (Jain, 1982). Heritability in the broad sense was estimated for eighteen quantitative characters using the formula

$$\text{Heritability } (h^2) = \frac{V_{(G)}}{V_{(P)}} \text{ or } \frac{V_{(G)}}{V_{(P)}} \times 100 \text{ (in percentage)}$$

4. Genotypic and phenotypic correlation

Correlation is a statistical technique used to measure and describe the simultaneous variation between two or more variables. The value of relationship between two variables is expressed as the correlation coefficient (r_{xy})

$$r_{xy} = \frac{CoV_{(xy)}}{\sqrt{V_{(x)} \times V_{(y)}}}$$

where r_{xy} is the estimate of sample correlation coefficient between 'x' and 'y'. $CoV_{(xy)}$ is the covariance between 'x' and 'y', which is a measure of the combined variance of the two characters 'x' and 'y'. $V_{(x)}$ is the variance of 'x' and $V_{(y)}$ is the variance of 'y'.

The genotypic and phenotypic correlation coefficients were calculated by replacing the variance and covariance tables of the above equation by the genotypic and phenotypic variance and covariance. These qualities were obtained from the analysis of the variance and covariance tables of any two characters. The significance of the correlation coefficients was tested with reference to the critical value of 'r' at (n-2) degrees of freedom, where 'n' is the number of pairs of observations used (Snedecor and Cochran, 1980).

5. Divergence analysis

The multivariate analysis using Mahalanobis D^2 statistics was used to group the cultivars. This is a useful technique for measuring the diversity in the available germplasm. Quantitative characters were used for the analysis.

A measure of group distance based on multiple characters was given by Mahalanobis (1928). Mahalanobis D^2 statistic is defined as follows

$$pD^2 = b_1 d_1 + b_2 d_2 + \ldots\ldots\ldots\ldots bp\ dp$$

where b_i (i = 1, 2,.....p) values are to be estimated such that the ratio of the variance between populations to the variance within population is maximized.

(b) Epidermal studies

Epidermal studies were carried out on all the sixty cultivars of *Hibiscus rosa-sinensis* following the methodology standardized by Ahmed (1964). Epidermal peelings were taken from both upper and lower surfaces of fresh leaves using a sharp razor blade. The peelings were then washed in distilled water and stained in aqueous 1% safranine for two minutes. The excess stain was washed away using distilled water. The peelings were then mounted in 50% glycerine, sealed using wax and observed under a binocular research microscope. The following epidermal characters were studied.

(i) Distribution of stomata

(ii) Shape of epidermal cell

(iii) Nature of epidermal cell wall

(iv) Type of stomata

(v) Number of subsidiary cells

(vi) Length of stomatal complex (µm)

(vii) Breadth of stomatal complex (µm)

(viii) Stomatal frequency

(ix) Stomatal index

(x) Stomatal abnormalities

(xi) Types of trichomes

The terminology proposed by Metcalfe and Chalk (1950) for stomata and Rao and Ramayya (1977 b) for trichomes were adopted in the present study for the classification of stomata and trichomes respectively.

The frequency of stomata was calculated as the number of stomata per unit area (10x X 40x field of the binocular research microscope) by taking the mean from 10 fields. The measurements of the stomatal size were taken using an ocular micrometer. All the measurements were taken from an average of 25 readings. The stomatal index was calculated using the formula

$$SI = \frac{S}{E+S} \times 100$$

where E= number of epidermal cells per unit area and S= number of stomata in the same unit area. Photomicrographs of the stomata were taken using an Olympus BH-2 research microscope. The photomicrographs of the trichomes were taken using a BX 51 H 02 Olympus trinocular research fluorescent microscope with image analyzer system.

The quantitative characters pertaining to the lower epidermis were analysed statistically to study the extent of variability among the cultivars

with regard to these characters, using analysis of variance, heritability, coefficient of variation and phenotypic and genotypic correlation coefficients.

B. Palynological study

Polliniferous materials were collected from live plants and fixed in glacial acetic acid. Pollen preparations were made by the acetolysis method standardized by Erdtman (1952) outlined as below.

The polliniferous materials in the glacial acetic acid were crushed with glass rods and sieved through a brass mesh with 48 divisions per square centimeter, into centrifuge tubes of 20ml capacity. The contents in the centrifuge tubes were centrifuged at 2400 rpm for 3 minutes. The supernatant was decanted and to each tube 5ml of freshly prepared acetolysis mixture (9 acetic anhydride: 1 concentrated sulphuric acid) was added. The centrifuge tubes were then heated in a water bath at 70^0C, repeatedly stirring the contents with glass rods. After 20 minutes, when the pollen turned light brown, the tubes were taken out and allowed to cool. The contents were then centrifuged at 2400 rpm for 3 minutes and the supernatant decanted. This was followed by the addition of 5 ml of glacial acetic acid to each tube. The mixture was then centrifuged for 3 minutes at 2400 rpm and the supernatant decanted. The sediment was then washed thrice in distilled water as was standardized by Nair (1970).

The acetolysed pollen were stored in 1:1 glycerine and distilled water for two weeks to attain a lighter shade of brown. The contents were then centrifuged, the glycerine decanted and the tubes containing the sediment

were placed upside down on a filter paper to drain off the excess glycerine. Permanent slides for light microscopic studies were prepared by mounting the acetolysed pollen in glycerine jelly and sealing with paraffin wax.

For SEM studies, a portion of the acetolysed pollen were preserved in 70% alcohol, dried and mounted on specimen studs. They were vacuum coated with gold using a JEOL – JFC – 1200 Fine Coater (Walker, 1974), and observed under the scanning electron microscope.

Morphological features of pollen grains including those relating to the aperture, exine ornamentation, pollen size and shape were studied from LM and / or SEM observations. The characters relating to the aperture have been considered primary, those of exine ornamentation secondary and other characters including size and shape tertiary (Nair, 1974). For ascertaining the shape class, the classification suggested by Erdtman (1966) was followed. The pollen grains were grouped into different size classes following Walker and Doyle (1975) as shown below

Class	**Longest axis**
Minute grain	<10 µm
Small grain	10-24 µm
Medium sized grain	25-49 µm
Large grain	50-99 µm
Very large grain	100-199 µm
Gigantic grain	≥ 200 µm

For describing the aperture types and exine ornamentation pattern of the pollen grains, the terminologies suggested by Punt *et al.* (1994) have been used.

Using an ocular micrometer, the measurements of the following characters were taken. In each case, the mean values of the measurements were taken from a random sample of 30 pollen grains.

(i) Diameter of pollen grain (µm)

(ii) Thickness of exine (minus spine) (µm)

(iii) Number of spines per unit area

(iv) Length of spine (µm)

(v) Width of the spine base (µm)

(vi) Interspinal basal distance-basal (µm)

(vii) Interspinal distance-apical (µm)

(viii) Number of apertures / unit area

(ix) Interporal distance (µm)

(x) Morphism type

In the case of cultivars exhibiting polymorphic grains, the measurements from the largest size class of grains were used for statistical analysis. Photomicrographs of the pollen preparations were taken for all the sixty cultivars using an Olympus BH-2 photomicroscope. SEM pictures of a few selected taxa (15) were taken at the Regional Research Laboratory, Pappanamcode, Thiruvananthapuram using a JEOL–JSM–5600LV scanning

electron microscope. The slides of pollen preparation are deposited in the Department of Botany, S.N. College, Kollam.

A detailed statistical analysis was conducted for the various palynological characters to assess the variability and correlation between them. The study included analysis of variance, heritability, coefficient of variation and phenotypic and genotypic correlation coefficients.

Hierarchical cluster analysis

The morphological, epidermal and palynological characters collected from all the sixty cultivars included in the present study were pooled together, standardized and subjected to Hierarchical cluster analysis (Johnson and Wichern, 2001) using the Average linkage study applying the SPSS package. A proximity matrix was prepared by calculating the squared euclidean distances between pairs of cultivars and a dendrogram was constructed illustrating the closeness of the relationship between the cultivars studied.

C. Molecular study

Sixteen representative samples of the fifteen clusters of *Hibiscus rosa-sinensis* obtained through the statistical analysis conducted based on the data collected from morphological, epidermal and palynological characters, were selected for the molecular study involving RAPD analysis. They include C-1, C-3, C-5, C-6, C-13, C-14, C-16, C-17, C-19, C-29, C-33, C-38, C-41, C-43, C-47 and C-55. The detailed procedure for the RAPD analysis is given

below. The expansions of the various abbreviations used, are given in Appendix I.

Genomic DNA extraction

Refrigerated High-speed Centrifuge (RC5C)

Water bath with temperature control

Glass centrifuge tubes

Eppendorf tubes

Pipettes

pH Meter

Electronic Balance

Table 2 - Stock solutions required for genomic DNA extraction

Reagent	Composition	
Tris buffer pH 8.0		
	Tris HCl	
	(pH adjusted to 8.0 by adding con. HCl)	
	H_2O	
EDTA 0.5 M		
	Na_2EDTA	18.61 gm
	(0.5 M EDTA pH adjusted with NaOH beads)	
	H_2O	to make up to 100 ml.
CTAB Extraction buffer		
	CTAB 2% (w/v)	2 gm
	Tris buffer 100 mM , pH 8.0	10 ml 1M Tris
	Na_2EDTA 20 mM , pH 8.0	4 ml 0.5 M Na_2EDTA
	NaCl 1.4 M	8.2 gm
	PVP 1.2%	1.2 gm
	H_2O	to make up to 100 ml
	β-Mercaptoethanol 1 %	1 ml.

CTAB NaCl

CTAB 10%	10 gm
NaCl 0.7 M	4.1 gm
H_2O	to make up to 100 ml

CTAB Precipitation buffer

CTAB 1 % (w/v)	1 gm
Tris buffer 50 mM, pH 8.0	5 ml (from stock)
Na_2EDTA 10 mM, pH 8.0	2 ml (from stock)
H_2O	to make up to 100 ml

High salt TE buffer

Tris buffer 10 mM, pH 8.0	1 ml 1 M Tris
Na_2EDTA 0.1mM, pH 8.0	20µl 0.5M Na_2EDTA
NaCl 1 M	5.85 gm
H_2O	to make up to 100 ml

1XTE

Tris buffer 10 mM, pH 8.0	1 ml 1 M Tris
Na_2EDTA 0.1mM, pH 8.0	20µl 0.5M Na_2EDTA
H_2O	to make up to 100 ml

RNase

RNase (pancreatic)	20 mg
Tris HCl 0.1 M, pH 7.5	200 µl
NaCl 1 M	30 µl
H_2O	to make up to 2 ml.

Quantification of DNA

UV-Visible Spectrophotometer (UV vos (2100), Shimadzu)

Quartz cuvette.

Sterile distilled water.

Reagents

TE buffer (1x)

RAPD

PCR –DNA Thermal Cycler 480 (Perkin Elmer)

PCR tubes

Reagents

1. RAPD primer kit (Kit P series, Operon technologies, USA)
2. PCR reagents; *Taq* Polymerase (Finnzymes, Bangalore.)
3. Sterile distilled water

Agarose gel electrophoresis

Electrophoresis system (Horizontal) (Bangalore Genei Pvt. Ltd., Bangalore.)

Electronic Balance

Pipettes

Gel documentation and analysis system: Alpha ChemiImager2000.

Table 3 - Stock reagents required for agarose gel electrophoresis

Reagent	Composition	Quantity
Tris Borate-EDTA buffer (10x)	Tris base	10. 80gm
	Boric acid	5.50 gm
	Na_2EDTA 0.5 M	4 ml
	H_2O	to make up to 100 ml.
Gel loading dye	Bromophenol Blue 0.25%	250 mg
	Xylene Cynaol 0.25%	250 mg
	Sucrose 40% (w/v)	40 gm
	H_2O	to make up to 100 ml
	Store at 4° C	
Ethidium bromide solution (stock)	Ethidium bromide	10mg
	H_2O	1 ml

Genomic DNA extraction

Standardization of protocol

Total genomic DNA from the young leaves of the plants was isolated following modified Murray and Thompson's method (1980) using CTAB. 1.2 % PVP-40T (MW 40,000 Sigma, USA) was added to the extraction buffer to remove phenolic contaminants and double $CHCl_3$ extraction at 10000 rpm helped to remove polysaccharides (Couch and Fritz, 1990). The DNA after ethanol precipitation was resuspended in 100 µl of 1x TE buffer.

Following this procedure, genomic DNA was isolated from the cultivars of *H. rosa-sinensis*. Quantitative estimation and purity analysis of DNA was determined spectrophotometrically.

Protocol

Step 1-Extraction

1. About 3gm of young leaf tissues were collected from the plant and washed thoroughly using distilled water and blot dried with tissue paper. It was then grinded in liquid nitrogen to a fine powder using a mortar and pestle.
2. To this, warm extraction buffer kept at 65° C with 1% β-Mercaptoethanol was added (4 ml for 1 gm of leaf tissue) and grinded well.
3. The slurry was then transferred to a screw capped 50ml centrifuge tube and incubated at 65° C for 3 hrs.
4. After incubation, $CHCl_3$ extraction was performed by adding equal volume of $CHCl_3$: octanol (24:1) mixture to the slurry and centrifuged at 10000 rpm for 5 min at 4°C to collect the supernatant.

5. To the supernatant 1/10 volume CTAB/NaCl and equal volume of $CHCl_3$ were added and centrifuged at 10000 rpm for 5 min at 4°C to collect the supernatant.

6. To the clear supernatant obtained, an equal volume of CTAB precipitation buffer was added and incubated overnight at 37° C in a water bath.

Step 2-Precipitation

7. On the next day the mixture was centrifuged at 8000 rpm for 10 minutes at 4° C to collect the precipitate.

8. The pellet collected was then resuspended in 1ml of high salt TE and transferred to a glass tube.

9. After transferring to glass tube 1ml of isopropanol was added to precipitate nucleic acids as a white ball by gentle shaking.

10. The tube was then sealed using tape and kept at -20° C for 30 minutes.

11. After incubation the tubes were taken out and centrifuged at 8000 rpm for 10 min at 4° C to collect the pellet .

12. The pellet collected was washed with 80% ethanol by spinning at 10000 rpm for 5 min and collected the pellet.

13. The pellet was resuspended in 0.5 ml 1x TE, shaken well and was transferred to an eppendorf tube.

14. 3 µl RNase was added to it, shaken well and kept for incubation at 55° C for 15 minutes.

15. After incubation 0.5 ml of $CHCl_3$ was added and centrifuged at 10000 rpm for 5 min.

16. The upper aqueous portion was carefully pipetted out in to a new eppendorf tube and more than double the volume of 100% ethanol and 50 µl of 3 M sodium acetate were added and kept at -20° C overnight, to precipitate DNA.

Step 3-Purification

17. On the next day the tube was taken out and centrifuged at 12000 rpm for 15 min to collect the pellet.

18. The white pellet collected was washed with 70% ethanol by spinning at 10000 rpm for 5 min and the supernatant discarded.

19. The pellet was then air dried to remove the alcohol and resuspended in 100 µl 1x TE buffer and stored at -20° C.

Quantification of DNA

Two types of methods are widely used to measure the amount of nucleic acids in a preparation. If the sample is pure (ie, without significant amount of contaminants such as proteins, phenol, agarose or other nucleic acids), spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases is simple and accurate .If amount of DNA or RNA is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted as against standard known molecular weight DNA markers (Sambrook *et al.*, 1989).

Spectrophotometric determination of the amount of DNA

The quantification of DNA was made from UV absorbance spectrophotometric readings taken at wavelengths of λ_{260} and λ_{280}.The readings at 260nm allow calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50µg/ml of double-stranded DNA, 40µg/ml of single stranded DNA, and 20µg/ml for single stranded oligonucleotide. The ratio between the readings at 260nm and 280nm(OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0 respectively. If there is contamination with protein or phenol, the OD_{260}/OD_{280} will significantly differ than the values given above, and accurate quantification of the amount of nucleic acid will not be possible.

From the stock DNA, diluted DNA samples were prepared for RAPD analysis. Based on the concentration, the DNA was diluted to 10 /100 times. The diluted DNA after tap spin was kept in a cryogenic box at -20°C.

RAPD

Standardization of protocol

RAPD assay was carried out in 25µl reaction mixture containing 2.5 µl 10x amplification buffer (10 mM Tris HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01%gelatin from Finnzymes), 0.5 µl of dNTP mixture (100µl each of dATP, dGTP, dCTP and dTTP), 0.5 µl Taq DNA polymerase (Finnzymes), 1.2 µl (15pM) of decamer primer (Operon Technologies Inc., USA) and 50ng of genomic DNA. The reaction mixture was overlaid with 15 µl of mineral oil in

order to avoid evaporation. Amplification was performed in DNA Thermal Cycler 480 (Perkin Elmer, USA).

Sequential steps involved

One cycle of

2 min at 95 °C

2 min at 35 °C

2 min at 72 °C

followed by 37 cycles of

1 min at 94 °C

1 min at 36 °C

2 min at 72°C

The last cycle was followed by 10 min extension at 72 °C.

Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying 0.5-25kb DNA fragments .The protocol can be divided into three stages

1. A gel is prepared with agarose concentration appropriate to the size of the DNA fragments to be separated.
2. The DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation.
3. The gel is stained with Ethidium bromide (EtBr) and visualized directly under UV illumination.

Preparation of gel

1. Adequate volume of electrophoresis buffer of working concentration (1x) was prepared to fill the electrophoresis tank and to prepare the gel.

2. Desired amount of electrophoresis grade agarose (Gibco-BRL) was added to a volume of 1x buffer sufficient for making a 1.2% gel, which can be used to separate DNA fragments of 400 bp to 7 Kb.

3. 2.4 gm of agarose was melted with 150 ml distilled water in a microwave oven. 20 ml of 10x TBE was added to it and the mixture made up to 200 ml. The melted agarose was allowed to cool to 40 °C and 10 µl of Et Br was added to it.

4. Then the melted agarose with EtBr was poured in to a horizontal gel-casting platform and a comb, which can produce sufficient number of wells to load all the samples, was inserted at one side.

5. After the gel was hardened, the comb was gently removed without tearing the wells. The sealing of cello tape along the sides of the gel-casting platform was also removed.

6. The gel-casting platform along with the gel was placed in the horizontal gel apparatus (Bio-Rad, USA) and sufficient amount of 1xTE buffer pH 8.0 was added to cover the gel to a depth of 1mm.

7. DNA samples mixed with 5 µl of 10x loading dye was then loaded into the wells using a micropipette.

8. After loading all the samples to the wells the electrophoresis apparatus was closed and the desired voltage ($10V/cm^2$) was applied through the leads.

9. When the bromophenol blue dye has migrated a distance sufficient for the separation of DNA fragments, the power supply was switched off.

10. As EtBr has been incorporated in the gel, DNA bands were visualized and documented using an Alpha ChemiImager Gel documentation system. Amplified products that were reproducible and consistent in performance were chosen for data analysis.

Estimation of genetic variation

POPGen 32, a computer program, was used to estimate standard genetic variability measures from data obtained from RAPD assay. The presence and absence of amplicons in the gels were scored as 0 and 1 respectively for the RAPD data analysis. Nei's original measures of genetic identity and genetic distance between cultivars were calculated from this data based on the percentage of common fragments (Nei and Li, 1979) according to the following equation

$$\text{Similarity} = 2\,Nab / (Na + Nb)$$

where Nab is the number of scored amplification products shared between accessions a and b. Na is the number of scored fragments in accession 'a' and Nb is the number of scored fragments in accession 'b'. Cluster analysis using the unweighed pair group method with arithmetic averages (UPGMA) was applied to construct a phenogram.