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Molecular characterization and genetic diversity determination of Hibiscus species using RAPD molecular markers

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ABSTRACT

The objective of this study was to find out the genetic relationship within the 9 varieties of Hibiscus rosasinensis through random amplified polymorphic (RAPD) markers. Genetic analysis was made by using 2 arbitrary decamer primers OPA9 and OPD10. The genetic similarity was evaluated on the basis of presence or absence of bands. High degree of polymorphism was observed among the samples, suggesting the degree of genetic variability. The genetic distance was very close within the varieties. RAPD analysis in combination with morphological characters can be used in the identification and determination of the genetic variation between the different varieties and species of Hibiscus. RAPD technique can be said to be reliable and promising for the characterisation of the Hibiscus germplasm and therefore Sequence Characterised Amplified Regions (SCAR) primers can be easily designed for many of these Hibiscus varieties and species. Thus, these RAPD markers have the potential for identification and characterization of genetic variation within the varieties in a species. This may also helpful in Hibiscus breeding programs and provides a major input into conservation biology.

Keywords: *Hibiscus species,* PCR, Genetic variations, Polymorphism, Dendrogram

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INTRODUCTION

Hibiscus rosasinensis is a species of flowering plant in the family Malvaceae, native to East Asia and it is known as rose mallow, Chinese hibiscus, China rose and shoe flower. *Hibiscus rosasinensis* is a bushy, evergreen shrub or small tree growing 2.5–5 m (8–16 ft) tall and 1.5–3 m (5–10 ft) wide, with glossy leaves and solitary, brilliant colour flowers in summer and autumn [1]. The genus contains about 300 species that grow in tropical and subtropical regions throughout the world [2]. It is widely grown as an ornamental and medicinal plant throughout the tropics and subtropics. Numerous varieties, cultivars, and hybrids are available, with flower colours ranging from white through yellow and orange to scarlet and shades of pink, with both single and double sets of petals. Some of the species are economically important as a source of food, beverage, fiber, medicines and other species as ornamentals [3, 4, 5]. *H. rosasinensis*, the tropical *Hibiscus*, has glossy heavy foliage with large, brilliant and spectacular flowers. A large number of hybrids and mutants are also grown in many parts of the world as well as in Indian subcontinent. A large number of varieties with spectacular flowers have been evolved but they are not properly documented. Particularly for the floricultural crops, morphological characteristics such as flower shape, size and colour were used to discriminate the species or varieties.

Until now in India, identification and classification of *Hibiscus* have mainly been based on morphology and according to [6] even if these descriptors are useful, they show limited levels of inter and intra-varietal

polymorphism and hence, may not account for all the diversity in the species. Since it is difficult to identify cultivars based entirely on these morphological features, several kinds of methods which can be used to measure levels and patterns of it is important to find an effective method to accurately identify the varieties to meet research needs. Modern molecular techniques have been developed in order to meet the demands of the horticulture industry genetic variation, which range from morphological characterization to various DNA-based markers such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) [7, 8, 9, 10, 11, 12]. Identification and characterization of germplasm is essential for the conservation and utilization of plant genetic resources [13]. Characterization of plant with the use of molecular markers is an ideal way to conserve plant genetic resources. Molecular characterization helps to determine the breeding behaviour of species, individual reproductive success and the existence of gene flow, the movement of alleles within and between populations of the same or related species, and its consequences [14].

Molecular data improves the elucidation of phylogeny, and provide the basic knowledge for understanding taxonomy, domestication and evolution of plants [15]. Random amplified polymorphic DNA (RAPD) technique has been widely used in many plant species for varieties analysis, population studies and genetic linkage mapping [16, 17, 18]. Optimization of the RAPD analysis depends on selection of primers. Although, the RAPD method uses arbitrary primer sequences, many of these primers must be screened in order to select primers that provide useful amplification products. The novelty of this project lies in the use of different molecular markers with increasing order of specificity to study genetic diversity which will help in development of new cultivars of *Hibiscus* varieties with superior properties to meet changing agronomic requirements.

This study was carried out to determine the genetic relationship between the 9 different varieties of *Hibiscus sp.* by using RAPD technique. This study will contribute basic knowledge in the aspect of their phylogenetic relationships and intra specific diversity.

MATERIALS AND METHODS

Sample collection:
Different varieties of *Hibiscus rosasinensis* leaf samples were collected from Lalbagh Botanical Garden, and local Nurseries in Bangalore, India. The leaves were collected based on colour and number of petals. The colour and number of petals of the flowers were Red - many petals (R1), Red- 5 petals (R2), pink- 5 petals, yellow with pink- 5 petals ,yellow- 5 petals, white with pink- many petals, white- 5 petals, orange with pink- many petals, orange- many petals. Fresh young leaves were plucked, placed in a labeled sterile plastic covers and immediately transported to the lab for processing. The samples were kept in a refrigerator at –20ºC until they were used for DNA extraction. Only healthy fresh green leaves were used for experimental analysis.

Genomic DNA Isolation:
The DNA was isolated and extracted according to the procedure described by [19]. 500 mg of the leaf material was well homogenized in 10 ml of STE buffer containing 4% of SDS, 0.04% of BSA 0.2% β- mercapto-ethanol. SDS, BSA and β- mercaptoethanol were added to the buffer immediately before use. The homogenization was done with sterilized mortar and pestle. LiCl (2 mM) was added to the homogenate before incubation at 65ºC for 30 min in a water bath. The incubated mixture was then left at room temperature for 10 min. Chloroform : isoamylalcohol (24:1, v/v) was added in an equal volume. The mixture was gently mixed and centrifuged at 15,000 rpm for 10 min at 4ºC. The upper phase was pipetted out in autoclaved centrifuge tube. The above step was repeated twice. Ice cold isopropanol was added in a ratio of 2:3 to the separated phase. The suspended DNA threads were spooled out with the help of fine capillary. The DNA was then washed with 70% ethanol, air dried and finally resuspended in TE buffer (pH 8).

Qualitative and Quantitative estimation of DNA:
DNA quality was assessed according to [20] by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm providing a value of 1.8 which determines pure DNA preparation.

The concentration of DNA in the sample was calculated using the given formula:

Concentration of DNA = A260× 50 µg× dilution factor

Purity of the DNA = A260: A280 ratio = A260 /A280

Quality of DNA fragment was electrophoretically analyzed through 0.8% agarose gel using 1X TAE buffer at 50 V for 45 mints. A 500 base pair ladder (purchased from Chromos biotech) was loaded into the gel as molecular size marker. The gel was visualized by staining with Ethidium bromide (1µl/10ml) and the bands were seen under UV light by using gel documentation system alpha imager hp (Innotech, USA).

RAPD PCR amplification:
RAPD PCR amplification was performed to amplify randomly unknown target sequences by using arbitary random primers according to the protocol described by [21]. PCR was carried out in a 25µl reaction volume containing 2.5µl of 10X Taq buffer, 1.5µl of dNTPs, 1µl of each primer, 2µl of Taq polymerase, 17 µl of water and 1µl of DNA (100 ng/ µl) for each sample in a Corbett Research CG1- 96 PCR Palm Cycler. OPA-6 (GGTCCCTGAC) and OPD-20 (ACCCGGTCAC) primers were used for RAPD amplification. The primers were purchased from Operon Technologies, USA. The thermal cycle profiles for 40 cycles were as follows denaturation at 94ºC for 1 min, annealing at 35ºC for 1 min, extension at 72ºC for 1 min. There was also an initial denaturation step for 5 min at 94ºC and, at the end of the 40 cycles, a final extension at 72ºC for 10 min and finally hold at 4ºC.

Separation and visualization of amplified products:
After amplification, PCR products were electrophoretically analyzed through 1.5% agarose gels, in 1X TBE buffer in a Protean II xi Cell (Bio-Rad, USA) Electrophoresis unit at 100 V for 90 minutes. l0 µl (6 µl of amplified sample and 4 µl of tracking dye) of sample was loaded into each well of the 1.0 mm thick gels.

Gels were stained with ethidium bromide and photographed under UV light by using gel documentation system alpha imager hp (Innotech, USA). The sizes of the amplified products were determined by comparison with a 500 bp ladder purchased from Chromos Biotech).

Analysis of RAPD data:
Data on the presence or absence of RAPD bands of identical molecular sizes were used for estimating genetic similarity coefficients. For all pairwise combinations, genetic similarity indices (SI) were calculated following the method of [22]. The formula for SI is given as $SI = 2.N_{AB}/(N_A+N_B)$, where N* is the number of RAPD bands shared in common between individuals A and B, and N_A and N_B are the total number of bands scored in A and B, respectively. The similarity matrix is calculated by Frequency similarity Index obtained from alpha imager hp gel doc software. Dendrogram for RAPD fragments were constructed by using an un-weighted pair group method of arithmetic mean of UPGMA.

RESULTS

Qualitative estimation of DNA by Agarose gel Electrophoresis
The quality of DNA extracted from different *Hibiscus* samples were analyzed by staining with Ethidium bromide and the bands were seen under UV light by using gel documentation system alpha imager hp (Innotech, USA). The single sharp bands in all the 9 lanes clearly indicated the presence of DNA in all samples without any RNA contamination (Figure 1).

Quantitative estimation of DNA by Nanodrop Spectrophotometer
Spectrophotometric analysis of the DNA samples showed the concentration of DNA obtained from *Hibiscus* samples in ng/µl were found to be 107.9, 120.6, 246.9, 138.7, 230.1, 339.3, 873.0, 124.7 and 324.5 respectively which indicates the presence of pure DNA

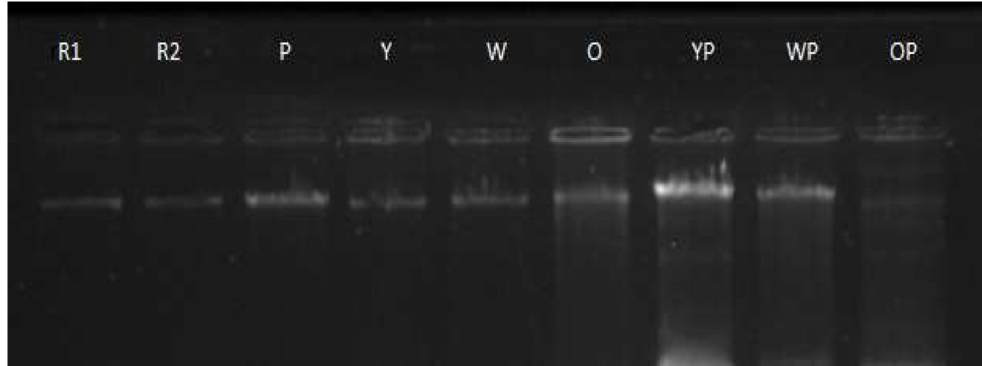

Figure-1: Isolated genomic DNA bands of various *Hibiscus* samples in the order: 1) Red many petals (R1), 2) Red 5 petals (R2), 3) Pink 5 petals (P), 4) Yellow 5 petals (Y), 5) White 5 petals (W), 6) Orange many petals (O), 7) Yellow with pink- 5 petals (YP), 8) White with pink- many petals (WP), 9) Orange with pink many petals (OP)

RAPD amplification

The RAPD profile of 9 different samples of *Hibiscus* were obtained by using OPA-6 (GGTCCCTGAC) and OPD-20 (ACCCGGTCAC) primers are as shown in (Figures 2 and 4). Number 1-9 represents different samples M is the molecular marker (500 bp) of low range DNA ladder. These two primers generated a total of 59 bands out of which 71.19% polymorphic, 20.34% monomorphic and 8.47% unique (Table 3).

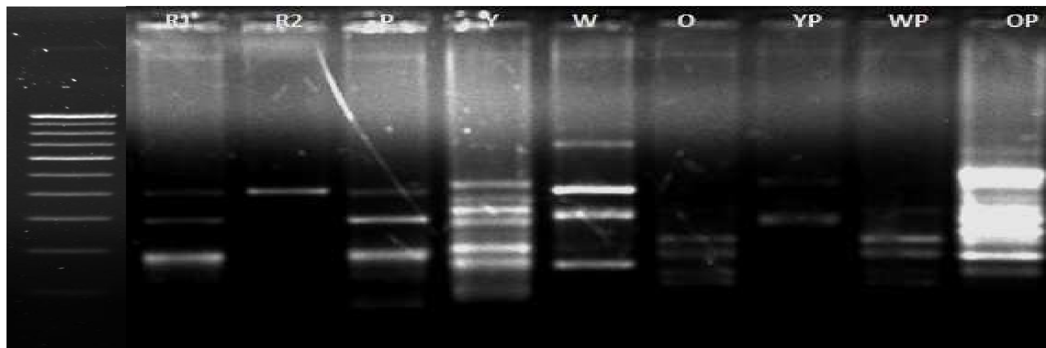

Figure-2: Random amplified polymorphic DNA fragment patterns generated using OPD- 20 primer

Table-1: Similarity Matrix Calculated by Frequency Similarity generated by using OPD- 20 primer

	1	2	3	4	5	6	7	8	9
1	**100.00**	81.25	100.00	81.25	12.50	93.75	93.75	75.00	62.50
2	81.25	**100.00**	81.25	62.50	31.25	75.00	75.00	68.75	56.25
3	100.00	81.25	**100.00**	81.25	12.50	93.75	93.75	75.00	62.50
4	81.25	62.50	81.25	**100.00**	18.75	87.50	87.50	68.75	56.25
5	12.50	31.25	12.50	18.75	**100.00**	6.25	6.25	25.00	25.00
6	93.75	75.00	93.75	87.50	6.25	**100.00**	100.00	81.25	68.75
7	93.75	75.00	93.75	87.50	6.25	100.00	**100.00**	81.25	68.75
8	75.00	68.75	75.00	68.75	25.00	81.25	81.25	**100.00**	75.00
9	62.50	56.25	62.50	56.25	25.00	68.75	68.75	75.00	**100.00**

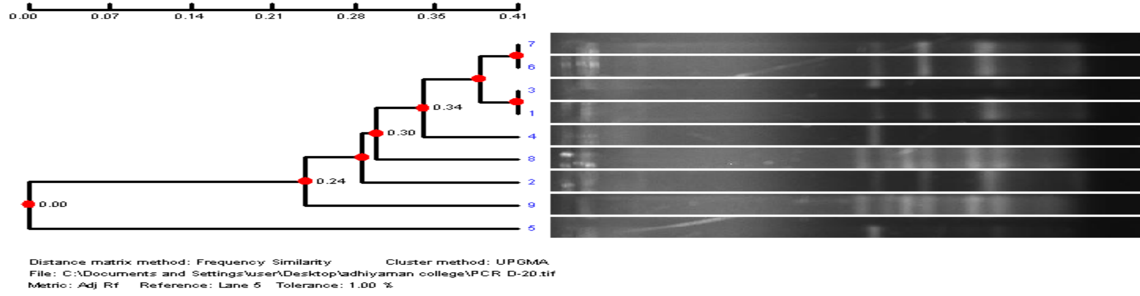

Figure-3: Dendrogram constructed by Similarity Matrix by using OPD- 20 primer

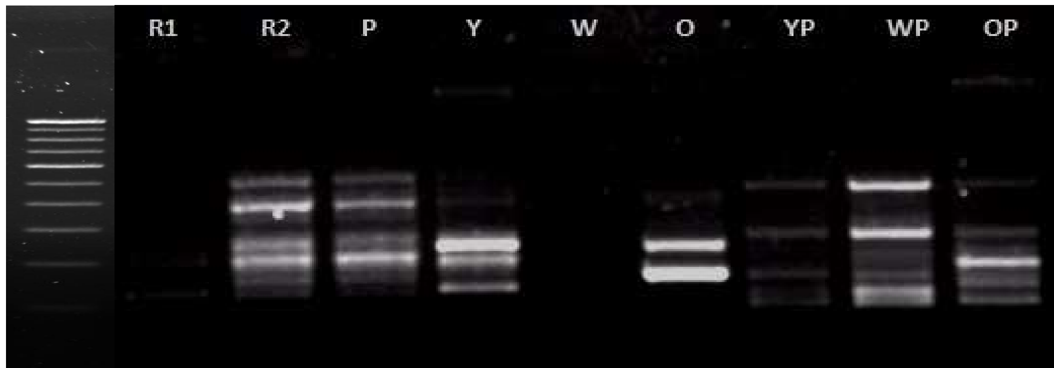

Figure-4: Random amplified polymorphic DNA fragment patterns generated using OPA-6 primer

Table-2: Similarity Matrix Calculated by Frequency Similarity generated by using OPA-6 primer

1	2	3	4	5	6	7	8	9	
1	**100.00**	50.00	50.00	83.33	66.67	66.67	16.67	66.67	83.33
2	50.00	**100.00**	66.67	33.33	50.00	50.00	33.33	50.00	66.67
3	50.00	66.67	**100.00**	66.67	50.00	50.00	66.67	50.00	33.33
4	83.33	33.33	66.67	**100.00**	83.33	83.33	33.33	83.33	66.67
5	66.67	50.00	50.00	83.33	**100.00**	100.00	16.67	100.00	83.33
6	66.67	50.00	50.00	83.33	100.00	**100.00**	16.67	100.00	83.33
7	16.67	33.33	66.67	33.33	16.67	16.67	**100.00**	16.67	0.00
8	66.67	50.00	50.00	83.33	100.00	100.00	16.67	**100.00**	83.33
9	83.33	66.67	33.33	66.67	83.33	83.33	0.00	83.33	**100.00**

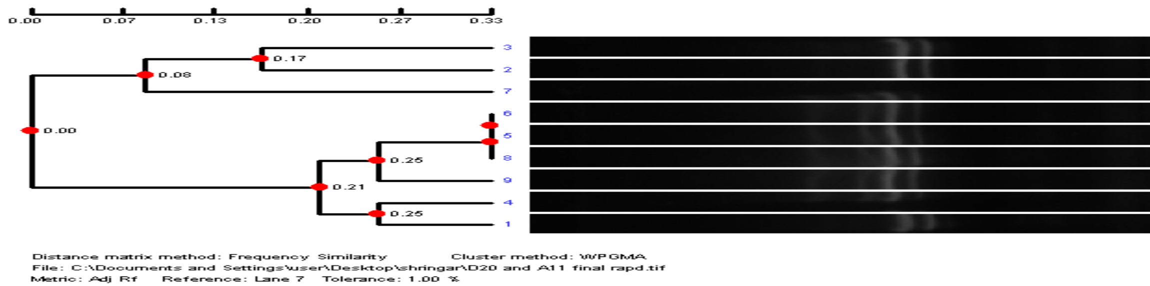

Figure-5: Dendrogram constructed by Similarity Matrix by using OPA-6 Primer

Table3: RAPD Pattern of 9 different *Hibiscus sp.* by using OPA-6 and OPD-20 primers

Pattern of polymorphism	OPA-6	OPD-20	Total
Total No. of bands	29	30	59
Total No. of polymorphic bands	23	19	42
Total No. of monomorphic bands	4	8	12
Total No. of unique bands	2	3	5
Polymorphism, %	79.31	63.33	71.19
Monomorphism, %	13.79	26.67	20.34
Uniqueness, %	6.90	10.00	8.47

DISCUSSION

The genetic variation through RAPD markers has been highlighted in a number of ornamental plants including rose [23, 24], Vanda sp. [25], Pelargonium [26], and Ixora cultivars [27]. The present study deals with, establishing a phylogenetic relationship between various hibiscus species using RAPD markers. In this study five *Hibiscus sp.* were selected. The present findings showed that there was high degree of variations within the varieties of *H. rosasinensis* flower plants. Even though all the varieties of *H. rosasinensis* have the same DNA profile, there were somehow some bands that were different from the other. This could be attributed to the difference in the morphology of variety 'Pink Double', which has double petals form and larger bloom size. Close relationships was observed between the species using the RAPD primers. This study showed that morphological characterization provided a rapid and satisfactory means to differentiate between certain *Hibiscus* species. Identification of some species and varieties of *Hibiscus* can be problematic, since flower color, shape and form are the only characters which can be used to discriminate between the species. Due to the influence of environment, the quantitative traits obtained using descriptive statistics proved to be unreliable as they vary a lot between species. The UPGMA dendrogram was prepared based on the similarity matrix indicating the segregation of the *Hibiscus sp.* populations collected from Bangalore, India. The similarity matrix was obtained after multivariant analysis using Nei and Li's coefficient and is presented in Table-1 (OPD- 20) and Table-2 (OPA-6). The similarity matrix was then used to construct a dendrogram with the unweighted UPGMA method presented in Figure-3 (OPD- 20) and Figure-5 (OPA-6). The dendrogram shows two major clusters for OPD- 20. The first major cluster contains sample no 5(White 5 petals) and the other cluster contains the remaining. From the dendrogram based on the similarity matrix clearly depicted the closest relationship between sample no 1 (Red many petals, R1), and 3 (Pink 5 petals, P), and sample no 6 (Orange many petals, O) and 7 (Yellow with pink- 5 petals, YP).

On the other hand the dendrogram obtained from OPA-6 primer shows two major clusters. Sample no 2 (Red 5 petals, R2) and 3 (Pink 5 petals, P), sample no 4 (Yellow 5 petals, Y) and 9 (Orange with pink many petals, OP) and sample no 5 (White 5 petals, W), 6 (Orange many petals, O), and 8 (White with pink- many petals, WP) showed least variations within the species. Our results were similar to the findings of [28]. They also indicated a higher genetic similarity between the hybrid tea and the floribunda group than within each group. The close relationships within the cultivars of celery were also reported by using RAPD markers [29]. This indicates that the RAPD markers provide a more reliable method for identification of varieties/species than morphological characters.

CONCLUSION

The RAPD analysis in this study has proven to be useful in discrimination, characterization and differentiation of *Hibiscus sp.* plant varieties and clustering them according to their origin. Despite the enormous and similar discriminating potential of the two markers used they showed some differences in their discrimination capacities. This indicates that the RAPD markers provide a more reliable method for identification of varieties/species than morphological characters. Since, the banding pattern by RAPD was variable depending upon the primer and species. Genetic mapping of the *Hibiscus* genome will help in understanding their complex traits such as yield, size, colour, flavor and shelf-life. Data obtained from this experiment demonstrated that morphological analyses together with RAPD markers are useful for classification and indication of relationships among the different Hibiscus and can be useful for Hibiscus breeding program.

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