GENETIC ANALYSIS ON HIBISCUS SPECIES BY USING RAPD MARKERS

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Abstract
This study was undertaken for genetic analysis within the 7 varieties of Hibiscus rosa-sinensis by using RAPD markers. The method involves the extraction of DNA from leaves of Hibiscus rosa-sinensis and the yield of DNA ranged from 158-200µg and purity (ratio) was between 1.3-1.6 indicating minimum level of contaminating metabolites. Genetic analysis and relationship among seven Hibiscus rosa-sinensis germplasm were analyzed using Random Amplified Polymorphic DNA (RAPD). Total nine primers were used as: RPI-07, RPI-08, RPI-10, RPI-12, RPI-18, RPI-19, RPI-21, RPI-22 and RPI-23. No PCR product was generated through the primer RPI-23 indicating that no binding sites for this primer. In all the primers RPI-07, RPI-08, RPI-10, RPI-19 and RPI-21 are more suitable for the genetic variation analysis in Hibiscus species as it has shown maximum polymorphic bands. Primers RPI-12, RPI-18 and RPI-22 are weak in showing the polymorphic banding pattern for all species of Hibiscus rosa-sinensis. By using PAST and MEGA programme Consensus Phylogenetic tree generated based on genetic distances segregated the seven Hibiscus germplasm into two different clusters. 75% times of species Hibiscus rosa-sinensis Red S.P. and D.P. (Single petal, Scarlet and Double petal, Rubroplenus) clustered in a single cluster. 87% times the cluster of Hibiscus rosa-sinensis Pink varieties clustered with Hibiscus rosa-sinensis White varieties. The results of the present study indicated that the RAPD analysis could be utilized by breeders for further improvement of Hibiscus rosa-sinensis species.

Keywords: RAPD, S.P., D.P., GERMPLASM, PAST, MEGA

1. Introduction
Hibiscus rosa-sinensis can endure extreme heat and cold, poor soil environment, and its flowers are modest in size and color. H. rosa-sinensis, the tropical hibiscus, has glossy heavy foliage with large, brilliant and spectacular flowers. There are numerous varieties of H. rosa sinensis obtained either by hybridization or mutation breeding. The initial activities of DNA extraction and quantification can be used in a variety of settings with a wide range of organisms as the source of the DNA. Advances in molecular techniques increased the availability of different DNA-based markers, which have become efficient tools in conservation genetic studies. Random amplified polymorphic DNA (RAPD) is a simple and straightforward PCR-based technique, which uses arbitrary primers for amplification of discrete regions of genome.

Medicinal applications
- Root is demulcent and used for Cough.
- A decoction of root is used for venereal diseases and fevers.
- Fresh root juice is given for gonorrhoea and powdered root for menorrhagia.
- Leaves are emollient, aperient, anodyne and laxative.
- Leaves and Stem bark are used for abortion.
- Staminal column is diuretic used for Kidney trouble.
- Flowers are astringent, demulcent, emollient, refrigerant, constipating, hypoglycaemic, aphrodisiac, emmenagogue and used for treating alopecia, burning sensation in the body, diabetes, and menstrual.
- Disorders.
- Buds are used in treatment of vaginal and uterine discharges.
- Leaves and flowers are good for healing ulcers and for promoting growth and color of hair.

And our Present work is on the genetic analysis in this mainly focused on the isolation of good quality of DNA from selected Hibiscus species, PCR amplification of selected Hibiscus species for RAPD and Analysis of all Hibiscus species for genetic variability.
2. Material and Methods

2.1.1. Collection of material: The leaf samples of *Hibiscus rosa-sinensis* cultivars studied in this research were obtained from the School of Biotechnology medicinal garden of Rajiv Gandhi Proudyogiki Vishwavidyalaya, Bhopal campus, Madhya Pradesh. This plant has been identified in many places of India such as botanical garden Kolkata and department of Plant sciences, University of Madras, the record is maintained in the form of voucher’s and the voucher no. is 035 at University of Madras and also authenticated as *Hibiscus rosa-sinensis*.

2.1.2. Solutions: DNA Extraction Buffer: 100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2.0% (W/V) CTAB, 0.2% 2-Mercaptoethanol, Chloroform-Isomyl alcohol (24:1), TE Buffer: 10 mM Tris (pH 8.0), 10 mM EDTA and Ethidium Bromide 10 mg/ml.

Isolation of plant genomic DNA: 5g of frozen leaf material in liquid nitrogen to fine powder was taken in the mortar, homogenous mixture was prepared, transfer the powder to a 25ml of pre-warmed DNA extraction buffer. Incubate at 60°C for 1 hour. Add 15ml chloroform-isoamyl alcohol and mix. Spin at 15000 rpm (10 min.). Take out the aqueous phase to another tube, add 2/3rd volume of isopropanol and mix. (If aqueous phase is not clear than repeated it another time for clear aqueous phase). Loop out DNA. Wash in 70% Ethanol, 10Mm Ammonium acetate for 20 minutes. Dry the pellet and dissolve in 4.5 ml of TE buffer. The DNA solution was stored at -20°C. Finally the quality and quantity of DNA was verified by electrophoresis on a 1.2 percent agarose gel.

2.1.3. DNA Amplification Protocol: The PCR tubes were labeled and 1.0µl of template DNA (10ng/µl) were added to the bottom most portion of the PCR tube. Primer (2.0µl) was added then. The four different primer used, Double distiller sterile water (39.0µl) was added next. 2µl 10mM dNTPs) + 1µl Taq polymerase (3U/µl) + 5µl of 10X Taq buffer A was added as shown in table-2. PCR tubes were closed and placed in a thermal cycler for amplification programmed as shown in table-1. Then, DNA Electrophoresis is done on 2.0 percent (polysaccharide derivative of agar) stained with Ethidium bromide. The microscopic pores in the agarose gel acts as a molecular sieve. Running buffer, which contained Tris-HCl, boric acid and 0.5M EDTA (pH 8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 8µl of PCR product and 5µl of loading buffer (sucrose, xylene cyanol, bromo phenol blue) together. Electrophoresis was conducted at 50V for 2-3 hours and the gel was observed and photographed under UV-Transilluminator.

2.1.4. DNA Analysis: Bands were manually scored 1 for presence and 0 for the absence and the binary data were used for statistical analysis by using the software “PAST”. The sizes of the fragments (molecular weight in base pairs) were estimated by using 100 bp ladder markers, which was run along with the amplified products. A genetic dissimilarity matrix was calculated according to Squared Euclidean Distances which estimated all pair-wise differences in the amplification product and cluster analysis was done by wards method using variance algorithm.

PCR (polymerase chain reaction) technology had led to the development of several novel genetic assays based on selective DNA amplification. Protocol is also relatively quick and easy to perform. Because the RAPD technique is amplification based assay, only nanogram quantities of DNA are required. One of the strengths of this new assay is that they are more amenable to automation than conventional techniques. It is to perform and is preferable to experiments where the genotypes of large number of individual are to be determined at a few genetic loci. With this idea the experiment was undertaken to evaluate genetic variation and relationship of some *Hibiscus* cultivars by RAPD technique.

3. Results

In order to perform RAPD, the *Hibiscus* leaves of 7 different varieties were collected and used for the good quality of genomic DNA had been isolated. Extraction of DNA from leaves of *Hibiscus rosa-sinensis* and then DNA quantified through spectrophotometer then DNA was amplified by using PCR technique. The PCR product produced were run on 2% agarose gel and observed under UV- transilluminator and photographs were taken. The analysis of *Hibiscus* cultivars through morphological features is inefficient and inaccurate. This problem is further compounded by perennial nature of crop. The use of biochemical and genetical markers for the identification of varieties offer a viable alternative method but now at present several *Hibiscus* cultivars have many synonyms or genetic analysis based on phenotype is a function of heritability of the trait. Factors like environment, traits of multigenic and quantitative inheritance, or partial and incomplete dominance often
compound the expression of genetic traits. Many of this complication of a phenotype based assay can be overcome through direct identification of genotype with DNA-based genetic markers. PCR had led to the development of several novel genetic assay based on selective DNA amplification. RAPD is an amplification based assay experiment is performed with only nanogram of DNA.

One of the strength of this new assay is that they are more amenable to automation than conventional techniques. It is simple to perform and is preferable to experiments where the genotype of large numbers of individuals can be determined at a few genetic loci. With this idea the experiment was undertaken to evaluate genetic analysis and relationship of some Hibiscus cultivars by RAPD technique. Nine primers used, RPI-07, RPI-08, RPI-10, RPI-12, RPI-18, RPI-19, RPI-21, RPI-22 and RPI-23. No bands was generated in the primer RPI-23. Primer RPI-07 gives 7 different bands. In these 6 are polymorphic band and 1 are monomorphic bands. Primer RPI-08 gives 8 different bands. In these all bands 7 are polymorphic and 1 are monomorphic band. Primer RPI-10 gives 10 different bands. Out of these 8 bands are polymorphic and 2 monopomorphic band. Primer RPI-12 gives 3 different bands and in them all are polymorphic. Primer RPI-18 gives 3 different bands and in these 2 bands are polymorphic and 1 are monomorphic band. Primer RPI-19 gives 7 different bands and 6 is polymorphic and 1 is monomorphic. Primer RPI-21 gives 7 different bands and in them 2 is monomorphic and 5 is polymorphic. Primer RPI-22 gives 3 different bands and in them all are polymorphic. The 9 primers generated different bands with size ranging from 100-1000bp. No PCR product was generated through the primer RPI-23 indicating that no binding sites for this primer. Out of the 48 bands, 40 Scorable bands (83.34%) were found to be polymorphic and 8 bands (16.66%) were found to be monomorphic. These results gave an average of polymorphic bands per primer was shown in table-3.

The gel images were analyzed for presence and absence of bands and this data was plotted as binary matrix to be used for phylogenetic analysis using PAST software. PAST (PAleontological STatistics) free software tool, runs on standard Windows computers. PAST integrates spreadsheet-type data entry with univariate and multivariate statistics, curve fitting, time-series analysis, data plotting, and simple phylogenetic analysis. Bootstrapping can be performed with a given number of replicates. PAST can read and export files in the NEXUS format. Here tree were generated by PAST program and visualization using MEGA tool with some editing of tree file to convert it to Newick Trees format.

3.1. Diversity analysis by Primer RPI-07:
Table-1 shows the Jaccards similarity coefficient matrix and Fig-1 is the dendogram generated using Jaccard’s similarity coefficient for the gel banding patterns generated by Primer RPI-07. Fig.-1: Phylogenetic tree generated from the banding pattern by Primer RPI-07: Primer RPI-07 shows the 100% similarity between the sub-varieties of Hibiscus species Red flower (Single petals and Double petal) shows similar pattern in the sub-varieties of Pink (Single petals and Double petal) variety of Hibiscus species. Jaccard’s similarity coefficient value is 1 for this pairs of sub-varieties. The Orange flower variety of Hibiscus is closer to White flower variety of Hibiscus with 83% similarity and 0.29 unit distance. The varieties Pink and Red flower are closer to each other than others with 50% similarity and 0.67 unit distance. This is clear from the table-4, Jaccard’s Similarity coefficient matrix. White and Red varieties shows major differences and hence are seems to be diversely related with the similarity of approx 30% and the distances were found 0.03 and 0.41 for the Hibiscus Red, Pink (S.P. and D.P.) and Orange and White (S.P. and D.P.) respectively.

Fig.-2: Phylogenetic tree generated from the banding pattern by Primer RPI-08:
Primer RPI-08 shows 100% similarity between the sub-varieties of Hibiscus species Red flower variety (Single petals and Double petal) and sub varieties of Hibiscus species pink flower variety (Single petal and Double petal) and also similar pattern in the sub-varieties of White flower variety of Hibiscus species. In table-5, Jaccard similarity coefficient value is 1 for this pairs of sub-varieties. The Orange flower variety of Hibiscus is closer to Pink flower variety of Hibiscus with 85% similarity and 0.13 unit distance. The varieties Pink and White diversely related to each other than others with 0.14 and 0.20 unit distance from Jaccard Similarity coefficient matrix.

Fig.-3: Phylogenetic tree generated from the banding pattern by Primer RPI-10:
Primer RPI-10 shows 100% similarity between the sub-varieties of Hibiscus species Red flower
variety (Single petals and double petal) diversely related shows similar pattern in the sub-varieties of White variety (Single petals and double petal) of *Hibiscus* species. In table-6, Jaccard’s similarity coefficient value is 1 for this pairs of sub-varieties. The Orange flower variety of *Hibiscus* is closer to Pink flower variety of *Hibiscus* with 90% similarity and 0.29 unit distance. The varieties Pink and white flower are not much closer to each other than orange with 0.22 and 0.20 unit distance similarity respectively.

**Fig.-4: Phylogenetic tree generated from the banding pattern by Primer RPI-12:**
Primer RPI-12 shows 100% similarity between the sub-varieties of *Hibiscus* species Red flower variety (Single petals and double petal) shows similar pattern in the sub-varieties of Pink variety (Single petals and double petal) diversely related of *Hibiscus* species. In the table-7, Jaccard’s similarity coefficient value is 1 for this pairs of sub-varieties. The Orange flower variety of *Hibiscus* is closer to White flower (Double petal) variety of *Hibiscus* with 0.33 unit distance similarity. The varieties Red and Pink (Single petals and Double petal) with White flower are not much closer to each other with 1.00 and 0.00 unit distance similarity respectively.

**Fig.-5: Phylogenetic tree generated from the banding pattern by Primer RPI-18:**
Primer RPI-18 shows Red (Double petals) species relates with the Red (Single petals) they are in a same group. Pink (S.P.) and Pink (D.P.) and White species both are showing 40% similarity they are diversely related. In the table-8, Jaccard’s similarity coefficient value is 1 for this pairs of sub-varieties. The Pink, Orange and White are not closer to the Red (D.P.) and Red (S.P.) flower species of *Hibiscus*. This is clear from the Jaccard’s Similarity coefficient matrix.

**Fig.-6: Phylogenetic tree generated from the banding pattern by Primer RPI-19:**
Primer RPI-19, showing perfect results on the similarity basis Red, Pink and White (Single petals and double petal) all are showing 100% similarity of 0.33 unit distances. In the table-9, Jaccard’s similarity coefficient value is 1 for this pairs of sub-varieties. The Orange flower variety of *Hibiscus* with 0.62 unit distances is not closer to Red, Pink and White flower (Single petals and Double petal) variety with 0.29 unit distances of similarity in *Hibiscus*. This is clear from the Jaccard’s Similarity coefficient matrix.

**Fig.-7: Phylogenetic tree generated from the banding pattern by Primer RPI-21:**
Primer RPI-21 shows 100% similarity between the sub-varieties of the species White and Pink flower with 0.57 unit distances with orange species of *Hibiscus*. The Red species with sub-varieties showing quite resemblances of 0.70 unit distances. Orange species shows 0.57 unit distance similarity with the other species of *Hibiscus*. In the table-10, of Jaccard’s Similarity coefficient matrix that are showing 100% similarity between White and Pink and Red sub-varieties.

**Fig.-8 Phylogenetic tree generated from the banding pattern by Primer RPI-22:**
Primer RPI-22 shows 100% similarity between the sub-varieties of the species White S.P and D.P. (Single petal and Double petal) flower shows 100% similarity. The Red species with sub-varieties showing quite resemblances of 0 unit distances. Orange species shows 0 unit distance similarity with the other species of *Hibiscus*. In the Jaccard’s Similarity coefficient matrix all rows and columns contains zero, showing 100% similarity with 0% dissimilarity.

**Fig.-9: Consensus Phylogenetic tree based on Phylogenetic tree given in Fig 1 to fig 8:**
This is the overall Consensus Phylogenetic tree which is based on Phylogenetic tree generated from the banding pattern by different primers RPI-07, RPI-08, RPI-10, RPI-12, RPI-18, RPI-19, RPI-21, RPI-22 and RPI-23. Among all the dendrogram drawn by using different primers 75 times of varieties of Pink Double petals and Pink Single petals were clustered in one cluster also the dendrogram drawn by using different primers, 75% times of species Red (Single petal and Double petal) clustered in a single cluster 87% times the cluster of *Hibiscus* species Pink varieties are clustered with Hibiscus White varieties. Varieties of *Hibiscus* species Red flower formed a out-group for 75%.

### 4. Discussion

*Hibiscus* is the most popular medicinal plant and choicest flower of India and so an important flower crop of India. Due to the availability of various varieties of *Hibiscus* flower it is difficult to identify the pure variety on the basis of morphological characters because the White variety are showing slight dissimilarity on the basis of leaves and also the color. Since the *Hibiscus* plants will take months to grow, flower and only then the quality and purity of the variety can be identified. Furthermore conventional plant breeding techniques are insufficient to improve *Hibiscus* cultivars. Since to raise healthy disease free, improved flowers
and leaves with respect to their medicinal properties.

Keeping above problems in mind RAPD markers is an efficient tool which permits to obtain information on genetic similarity among Hibiscus plants. The main objective of this investigation is to isolate good quality genomic DNA of seven Hibiscus rosa-sinensis species for genetic analysis by using PAST and MEGA Bioinformatics tools.

The genomic DNA was isolated by using Doyle and Doyle, 1987 method. Isolated DNA was quantified and provided optimized conditions for PCR amplification appropriate reaction mixture using nine RAPD primers. The PCR products then, run on agarose gel electrophoresis. The bands are manually scored from amplification profile on gel. The binary data obtained is put in PAST programme. PAST can read and export files in the NEXUS format. Here tree were generated by PAST programme. I.Hammad (2009) support these programme. Visualization using MEGA tool with some editing of tree file to convert it to Newick Trees format.

Genetic analysis and relationship among seven Hibiscus germplasm were analyzed using Random Amplified Polymorphic DNA (RAPD). In all the primers RPI-07, RPI-08, RPI-10, RPI-19 and RPI-21 are more suitable for the genetic variation analysis in Hibiscus species as it has shown maximum polymorphic bands. Primers RPI-12, RPI-18 and RPI-22 are weak in showing the polymorphic banding pattern for the all species of Hibiscus rosa-sinensis.

Low primer concentration affect the weak polymorphic banding pattern as primer binding site is low and impure DNA with protein. The phenotypic differences observed in seven different species using nine primers generated 48 bands with size ranging from approx. 100-1000 bp. On an average 5.3 bands generated per primer. Out of the 48 bands, 40 Scorable bands (83.34%) were found to be polymorphic and 8 bands (16.66%) were found to be monomorphic. Consensus tree of eight phylogenetic trees of all 8 primer was generated using the method of majority rule consensus (Nei & Kumar S, 2000). At 50% cutoff Double and Single petal sub-varieties of Pink flower Hibiscus varieties are genetically very close to each other. The cluster analysis based on genetic distances shows that Pink (Single petal) and Pink (Double petal) shows the shortest genetic distances Similarly, White (Single petal) and White (Double petal) are also genetically similar to each other by using Jaccard similarity coefficient matrix. Dendrogram also shows Orange (Single petal) is less genetically related to the White and Pink species. Red (Single petal) and Red (Double petal) is most distantly related to the all other species. The results of the present study indicated that the RAPD analysis could be utilized by breeders for further improvement of Hibiscus species. The application of RAPDs and their related modified markers in variability analysis and individual-specific genotyping has largely been carried out, but is less popular due to problem such as poor reproducibility faint or fuzzy products, and difficulty in scoring bands, which lead to inappropriate inferences. RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers. It is observed that hibiscus varieties as Pink flower variety, White flower variety, Red flower variety and Orange flower variety formed separate cluster, indicating the genetic similarity at variety level. Pink and White flower varieties are genetically in close relation so clustered in one group whereas Red and Orange flower varieties are closer to each other. Double and Single petal flower sub-varieties of each color flower varieties are clustered together indicating genetical closeness of the genetic make-up. It can also be implied that the formation of different coloured varieties of flower are due to the early genetic changes than the single and double petal flower.

5. Conclusion

RAPD marker is an efficient tool which permits to obtain information on genetic similarity among Hibiscus plants. The main objective of this investigation is to isolate a good quality genomic DNA of four Hibiscus cultivars, standardization of PCR amplification protocol for RAPD and analysis of four Hibiscus species for genetic variability. The genomic DNA was isolated using CTAB method. Isolated DNA was quantified and provided optimized conditions for PCR amplification appropriate reaction mixture using 9 RAPD primers, the PCR product then run on agarose gel electrophoresis, the bands are manually scored from amplification profile on gel. In the Consensus Phylogenetic tree which is based on Phylogenetic tree generated from the banding pattern by different primers RPI-07, RPI-08, RPI-10, RPI-12, RPI-18, RPI-19, RPI-21, RPI-22 and RPI-23. Among all the dendrogram drawn by using different primers 75
times of varieties of Pink Double petals and Pink Single petals were clustered in one cluster also the dendrogram drawn by using different primers, 75% times of species Red (Single petal and Double petal) clustered in a single cluster 87% times the cluster of Hibiscus species Pink varieties are clustered with Hibiscus White varieties. Varieties of Hibiscus species Red flower formed an outgroup for 75%. Hibiscus species Pink varieties shows 100% similarity in a similar way the Hibiscus species White varieties are also shows 100% similarity and also the their genetic similarity are much closer to each other as compared to other Hibiscus species Orange and Red varieties. The present study on the development of protocol for isolation of high purity DNA and analysis by RAPD conditions may serve as an efficient tool for further molecular studies. The genetic distance was very close within the varieties and also among the species. Thus, these RAPD markers have the potential for the identification of species and varieties and characterization of genetic variation within the varieties. This is also helpful in Hibiscus breeding programs and provides a major input into conservation biology.

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Table 1: RAPD—“PCR Conditions”

| Temp (°C) | Temp (°C) | Temp (°C) | Temp (°C) | Temp (°C) | Temp (°C) |
|-----------|-----------|-----------|-----------|-----------|-----------|
| 94°C      | 94°C      | 35°C      | 72°C      | 94°C      | 38°C      |
| 5 min     | 45 sec    | 2 min     | 1.5 min.  | 45 sec    | 1 min.    |
| Denaturation | x8 cycles | x35 cycles | Final Extention |

Table 2: In a PCR reaction mixtures following components are added:

| Sr. No. | COMPONENTS                | AMOUNT |
|---------|---------------------------|--------|
| 1.      | Sterile water             | 39.0µl |
| 2.      | 10X Taq Buffer A          | 5.0µl  |
| 3.      | 10mM dNTPs mix            | 2.0µl  |
| 4.      | RAPD primer               | 2.0µl  |
| 5.      | DNA Template (10ng/µl)    | 1.0µl  |
| 6.      | Taq DNA polymerase (3U/µl)| 1.0µl  |
| 7.      | Total Volume              | 50.0µl |

Table 3: RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in seven Hibiscus germplasm.

| S.No | Primer codes | Accession Numbers | Total no. of bands scored | Size ranges (bp) | Number of monomorhic bands | Number of polymorphic bands | Proportion of polymorphic loci (%) |
|------|--------------|-------------------|---------------------------|------------------|---------------------------|-----------------------------|----------------------------------|
| 1.   | RPI-07       | AM773312           | 7                         | 100-900          | 1                         | 6                           | 86%                              |
| 2.   | RPI-08       | AM773773           | 8                         | 200-800          | 1                         | 7                           | 87.5%                            |
| 3.   | RPI-10       | AM750045           | 10                        | 100-1000         | 2                         | 8                           | 80%                              |
| 4.   | RPI-12       | AM773316           | 3                         | 100-900          | 0                         | 3                           | 100%                             |
| 5.   | RPI-13       | AM750046           | 3                         | 100-900          | 1                         | 2                           | 66.6%                            |
| 6.   | RPI-19       | AM773777           | 3                         | 200-900          | 0                         | 3                           | 100%                             |
| 7.   | RPI-21       | AM765820           | 7                         | 100-600          | 1                         | 6                           | 86%                              |
| 8.   | RPI-22       | AM911711           | 7                         | 100-900          | 2                         | 5                           | 71.4%                            |
| Total|              |                    | 48                        |                  | 8                         | 40                          | 83.3%                            |
| Average|           |                    | 6                         |                  | 1                         | 5                           | 10.4                             |
Different RAPD – PCR banding pattern of eight different species of *Hibiscus rosa-sinensis*.

**Fig.1:** RAPD banding pattern by Primer RPI-07

**Fig.2:** RAPD banding pattern by Primer RPI-08

**Fig.3:** RAPD banding pattern by Primer RPI-10

**Fig.4:** RAPD banding pattern by Primer RPI-12

**Fig.5:** RAPD banding pattern by Primer RPI-18

**Fig.6:** RAPD banding pattern by Primer RPI-19

**Fig.7:** RAPD banding pattern by Primer RPI-21

**Fig.8:** RAPD banding pattern by Primer RPI-22

### Table 4: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-07

|          | HIBI-RED-S.P. | HIBI-PINK-S.P. | HIBI-ORANGE-S.P. | HIBI-WHITE-S.P. | HIBI-RED-D.P. | HIBI-PINK-D.P. | HIBI-WHITE-D.P. |
|----------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI-RED-S.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI-PINK-S.P. | 0.33          | 1.00           | 0.33             | 0.33             | 0.33          | 1.00           | 0.33            |
| HIBI-ORANGE-S.P. | 0.14          | 0.33           | 1.00             | 0.71             | 0.14          | 0.33           | 0.33            |
| HIBI-WHITE-S.P. | 0.33          | 0.33           | 0.71             | 1.00             | 0.33          | 0.33           | 1.00            |
| HIBI-RED-D.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI-PINK-D.P. | 0.33          | 1.00           | 0.33             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI-WHITE-D.P. | 0.33          | 0.33           | 0.71             | 1.00             | 0.33          | 0.33           | 1.00            |

### Table 5: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-08

|          | HIBI-RED-S.P. | HIBI-PINK-S.P. | HIBI-ORANGE-S.P. | HIBI-WHITE-S.P. | HIBI-RED-D.P. | HIBI-PINK-D.P. | HIBI-WHITE-D.P. |
|----------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI-RED-S.P. | 1.00          | 0.14           | 0.13             | 0.20             | 1.00          | 0.14           | 0.20            |
| HIBI-PINK-S.P. | 0.14          | 1.00           | 0.88             | 0.71             | 0.14          | 1.00           | 0.71            |
| HIBI-ORANGE-S.P. | 0.13          | 0.88           | 1.00             | 0.63             | 0.13          | 0.88           | 0.63            |
| HIBI-WHITE-S.P. | 0.20          | 0.71           | 0.63             | 1.00             | 0.20          | 0.71           | 1.00            |
| HIBI-RED-D.P. | 1.00          | 0.14           | 0.13             | 0.20             | 1.00          | 0.14           | 0.20            |
| HIBI-PINK-D.P. | 0.14          | 1.00           | 0.88             | 0.71             | 0.14          | 1.00           | 0.71            |
| HIBI-WHITE-D.P. | 0.20          | 0.71           | 0.63             | 1.00             | 0.20          | 0.71           | 1.00            |

### Table 6: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-10

|          | HIBI-RED-S.P. | HIBI-PINK-S.P. | HIBI-ORANGE-S.P. | HIBI-WHITE-S.P. | HIBI-RED-D.P. | HIBI-PINK-D.P. | HIBI-WHITE-D.P. |
|----------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI-RED-S.P. | 1.00          | 0.25           | 0.33             | 0.22             | 1.00          | 0.25           | 0.22            |
| HIBI-PINK-S.P. | 0.25          | 1.00           | 0.71             | 0.67             | 0.25          | 1.00           | 0.67            |
| HIBI-ORANGE-S.P. | 0.33          | 0.71           | 1.00             | 0.44             | 0.33          | 0.71           | 0.44            |
| HIBI-WHITE-S.P. | 0.22          | 0.67           | 0.44             | 1.00             | 0.22          | 0.67           | 1.00            |
| HIBI-RED-D.P. | 1.00          | 0.25           | 0.33             | 0.22             | 1.00          | 0.25           | 0.22            |
| HIBI-PINK-D.P. | 0.25          | 1.00           | 0.71             | 0.67             | 0.25          | 1.00           | 0.67            |
| HIBI-WHITE-D.P. | 0.22          | 0.67           | 0.44             | 1.00             | 0.22          | 0.67           | 1.00            |
Table 7: Jaccard’s Similarity Matrix of gel banding pattern of Primer: RPI-12

|        | HIBI.-RED-S.P. | HIBI.-PINK-S.P. | HIBI.-ORANGE-S.P. | HIBI.-WHITE-S.P. | HIBI.-RED-D.P. | HIBI.-PINK-D.P. | HIBI.-WHITE-D.P. |
|--------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI.-RED-S.P. | 1.00          | 1.00           | 0.33             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-PINK-S.P. | 1.00          | 1.00           | 0.33             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-ORANGE-S.P. | 0.33          | 0.33           | 1.00             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-WHITE-S.P. | 0.00          | 0.00           | 0.00             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-RED-D.P. | 1.00          | 1.00           | 0.33             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-PINK-D.P. | 1.00          | 1.00           | 0.33             | 0.00             | 0.00          | 0.00           | 0.00            |
| HIBI.-WHITE-D.P. | 0.00          | 0.00           | 0.67             | 0.00             | 0.00          | 0.00           | 0.00            |

Table 8: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-18

|        | HIBI.-RED-S.P. | HIBI.-PINK-S.P. | HIBI.-ORANGE-S.P. | HIBI.-WHITE-S.P. | HIBI.-RED-D.P. | HIBI.-PINK-D.P. | HIBI.-WHITE-D.P. |
|--------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI.-RED-S.P. | 1.00          | 0.33           | 0.33             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI.-PINK-S.P. | 0.33          | 1.00           | 1.00             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-ORANGE-S.P. | 0.33          | 1.00           | 1.00             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-WHITE-S.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.67          | 1.00           | 1.00            |
| HIBI.-RED-D.P. | 1.00          | 0.33           | 0.33             | 0.33             | 1.00          | 0.33           | 1.00            |
| HIBI.-PINK-D.P. | 0.33          | 1.00           | 1.00             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-WHITE-D.P. | 0.33          | 1.00           | 1.00             | 1.00             | 0.33          | 1.00           | 1.00            |

Table 9: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-19

|        | HIBI.-RED-S.P. | HIBI.-PINK-S.P. | HIBI.-ORANGE-S.P. | HIBI.-WHITE-S.P. | HIBI.-RED-D.P. | HIBI.-PINK-D.P. | HIBI.-WHITE-D.P. |
|--------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI.-RED-S.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI.-PINK-S.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-ORANGE-S.P. | 0.14          | 0.43           | 1.00             | 0.43             | 0.14          | 0.43           | 0.43            |
| HIBI.-WHITE-S.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-RED-D.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI.-PINK-D.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-WHITE-D.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |

Table 10: Jaccard’s Similarity Matrix of gel banding pattern of Primer RPI-21

|        | HIBI.-RED-S.P. | HIBI.-PINK-S.P. | HIBI.-ORANGE-S.P. | HIBI.-WHITE-S.P. | HIBI.-RED-D.P. | HIBI.-PINK-D.P. | HIBI.-WHITE-D.P. |
|--------|---------------|----------------|------------------|------------------|---------------|----------------|-----------------|
| HIBI.-RED-S.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI.-PINK-S.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-ORANGE-S.P. | 0.14          | 0.43           | 1.00             | 0.43             | 0.14          | 0.43           | 0.43            |
| HIBI.-WHITE-S.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-RED-D.P. | 1.00          | 0.33           | 0.14             | 0.33             | 1.00          | 0.33           | 0.33            |
| HIBI.-PINK-D.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |
| HIBI.-WHITE-D.P. | 0.33          | 1.00           | 0.43             | 1.00             | 0.33          | 1.00           | 1.00            |

Fig. 1: Phylogenetic tree generated from the banding pattern by Primer RPI-07
Fig. 2: Phylogenetic tree generated from the banding pattern by Primer RPI-08

Fig. 3: Phylogenetic tree generated from the banding pattern by Primer RPI-10
Fig.4: Phylogenetic tree generated from the banding pattern by Primer RPI-12

Fig.5: Phylogenetic tree generated from the banding pattern by Primer RPI-18
Fig. 6: Phylogenetic tree generated from the banding pattern by Primer RPI-19

Fig. 7: Phylogenetic tree generated from the banding pattern by Primer RPI-21
Fig. 8: Phylogenetic tree generated from the banding pattern by Primer RPI-22

Fig. 9: Consensus Phylogenetic tree based on Phylogenetic tree given in Fig 1 to Fig 8.

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