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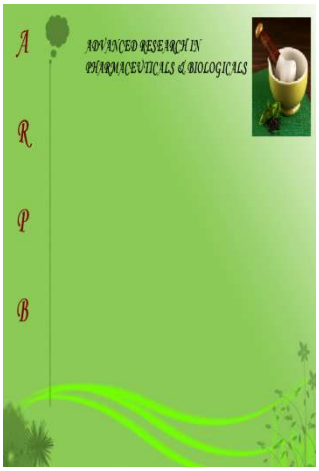

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GENETIC VARIATION ON HIBISCUS SPECIES BY USING RAPD MARKERS

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ABSTRACT:

Hibiscus rosa-sinensis is a attractive ornamental plant with brilliant red, double whorled crumpled red, orange, pink, double whorled crumpled pink and white flowers. This study was undertaken for genetic analysis within the four varieties of *Hibiscus* rosa-sinensis by using RAPD markers. The method involves a extraction of DNA from leaves of *Hibiscus rosa-sinensis* and yield of DNA ranged from 158-200µg and purity (ratio) was between 1.3-1.6 indicating minimum level of contaminating metabolites and the isolated DNA was used for RAPD analysis. Genetic analysis was made by using four primers of RP1, RP2, RP3 and RP4. RAPD results produced scorable banding pattern with different primer used in the experiment. From a total of 6 samples distinct DNA fragments ranging from 0.3 to 2.5 kb were amplified by using selected random decamer primers. On this basis genetic similarity was evaluated on the absence or presence of bands. 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/varieties and characterization of genetic variation within the varieties of species. This is also useful in *Hibiscus* breeding programs and provides a major input into conservation biology.

Keywords: Genetic Variation, Bands, RAPD.

INTRODUCTION

High quality DNA is required for molecular biological studies of plants in these different DNA extraction procedures including salt extraction and cetyl trimethyl ammonium bromide (CTAB) method[1] and Doyle and Doyle method[2] used for the isolation of genomic DNA. Germplasm identification and characterization is an important link between the conservation and utilization of plant genetic resources. Traditionally, species or cultivars identification has relied on morphological characters like growth habit or floral morphology like flower color and other characteristics of the plants[3]. RAPD has proven to be a useful tool for genetic typing and mapping[4]. PCR is the most important new scientific technology to come along in the last hundred years," says Mark R. Hughes, deputy director of the National Center for Human Genome Research at the National Institutes of Health (perhaps better known as the Human Genome Project).Science has pointed out that, because it is far simpler and less expensive than previous techniques for duplicating DNA, PCR has democratized genetic research, putting it within reach of all biologists, even those with no training in molecular biology[5].

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[6].

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.

REVIEW OF LITERATURE

Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, not require prior knowledge of a DNA sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate[7], on the otherhand, RAPD technique is used to evaluate the genetic diversity among five *Solanum* species RAPD analysis using five primers produced a total of 38 polymorphic bands and also in the solanum species number of polymorphic loci was 19 and the percentage of polymorphism was 50. The overall observed and effective number of alleles was 1.5000 and 1.3306, result indicates the presence of high level genetic diversity in *Solanum* species and a dendrogram constructed by UPGMA methods shows that *S. nigrum* is closely related to *S. surattense* followed by *S. trilobatum*. *Solanum torvum* shows more dissimilarity with other four species[8].

Nei's concluded the overall gene diversity was 0.1937[9]. Genetic diversity from 10 samples of C. occidentallis collected from Haryana (india) using 10 random primers, 111 bands were scored with an average of 9.25 bands per primer with 79 bands showing polymorphism (71.7%) 9 out of 12 primers gave more than 60% polymorphism[10]. By using RAPD technique five primers used in the *Steindachneridion melanodermatum* produced 53 fragments on gels, 15.1% of which were in monomorphic bands and 84.9% were in polymorphic bands. Fragment size ranged from 200 to 2000 bp, and the number of bands ranged from one to 16. Genetic similarity ranged from 0.57 to 0.95[11]. To avoid the problem of multiple allelic markers is to focus on markers that are common[12]. Random amplified polymorphic DNA can provide simple and reproducible fingerprints of germplasm by employing single, arbitrary chosen primers[13]. RAPD can also be used in monitoring diversity

within plant populations for constructing linkage maps and for tracking hybrid species origins[14]. Some findings suggest caution in making conclusions regarding genetic relationships of cultivars or selections within a species[15] and question the reproducibility of RAPD markers. However, in petunia (*Petunia hybrida* Vilm) and cyclamen (*Cyclamen persicum* Mill.), RAPDs were used successfully to test genetic purity of selected cultivars[16]. Reliable and reproducible RAPD assays were also reported for cucumber[17] and for rose cultivar fingerprinting, where by the use of eight primers, five cultivars were distinguished[18].

The best one was found to completely digest the genomic DNA and this was the combination of three restriction enzymes Eco RI, Bam HI, Hind III at 37ºC for 15-18 hours. The dual success in developing a standardized condition for restriction digestion of genomic DNA from this species as well as the obtaining of completely digested plant genomic DNA can be further used in DNA Analysis of the related cultivars, and their RAPD analysis can be carried out which helps to identify and characterize the plants within this species as part of germplasm conservation[19]. Analysis of the six ISSR primers among the turfgrass cultivars in this study generated 51 bands. Over 96% (49 bands) were found to be polymorphic in the turfgrass cultivars. The genetic similarity coefficients among all turfgrasses ranged from 0.43 to 0.88.

The dendogram constructed with UPGMA analysis revealed two main clusters[20]. The Callus derived from the somatic hybridization, RAPD banding patterns for the two Hibiscus species were developed DNA extracted from callus of both species, and 40 arbitrarily selected 10-base-long primers were used to generate RAPD products in 45 amplification cycles, polymorphic bands were obtained for OPD-2, OPD-4, OPD-16, OPF-1, OPF-3 and OPF-14 primers. It is expected that the markers will be suitable for identification of callus combining genomic DNA from both species hybridized[21].

Relationships among twelve species of *Phyllanthus* (India) by molecular markers. 259 marker loci were assessed, out of which 249 were polymorphic revealing 96.13% polymorphism. Cluster analysis by the unweighted pair group method (UPGMA) of Dice coefficient of similarity generated dendrogram with more or less similar topology for both the analyses that offered a better explanation for diversity and affinities between the species[22]. Genetic analysis was made by using 15 selected decamer primers and 9

selected ISSR markers. A total of 164 and 69 distinct DNA fragments ranging from 300 to 2500 bp were amplified by using selected random RAPD and ISSR primers respectively. The cluster analysis indicated that the 14 varieties/species of *Polyscias* formed one major cluster and *Schefflera elegantissima* forming another major cluster. The correlation matrix indicates that there was significant correlation between ISSR and RAPD markers[23].

Sixty-nine accessions representing wild and domesticated high bush blueberry *(Vacciniu, n corvrnbosum* L.) germplasm were genotyped using 28 simple sequence repeats (SSRs). A total of 627 alleles were detected and unique fingerprints were generated for all accessions. Suspected duplicate accessions of 'Coville' and 'Ivanhoe' had DNA fingerprints that were identical to 'Coville' and 'Ivanhoe', respectively. Genetic similarity measures placed wild and cultivated blueberries in separate groups[24]. Genetic diversity between fifteen cultivars of rose mainly 126 bands were produced, 73 of which were polymorphic, bands ranged from 37% to 81% with an average of 63.9%. The average number of polymorphic bands produced was 7.3 per primer, Cluster analysis based on the presence or absence of bands was performed by Jaccard's similarity coefficient, based on Unweighted Pair Group Method with Arithmetic Averages (UPGMA). Genetic similarity ranged between 0.12 to 0.53[25]. Studies were undertaken for identification and determination of genetic variation between four cultivars of *Hibiscus rosa-sinensis* L. with different colors (red, pink, orange and white) through inter-simple sequence repeat (ISSR) and isozymes pattern. ISSR result produced scorable banding patterns with nine primers out of ten. A total number of 89 DNA fragments were amplified with different lengths over all the four cultivars with the nine primers[26]. The Genetic similarities among the four *Ficus* cultivars were estimated according to the RAPD and ISSR data. Cultivars distribution on the consensus tree according to the banding patterns of RAPD differed from that based on ISSR[27].

POLYMERASE CHAIN REACTION (PCR)

PCR stands for the Polymerase Chain Reaction and was developed in 1987 by Kary Mullis (which won him a Nobel Prize) and associates. It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. It is technically difficult to amplify targets >5000 bp long[28].

The PCR reaction requires the following components:

Template: The quality of the template influences the outcome of the PCR. For instance, large amounts of RNA in a DNA template can chelate Mg^{2+} and reduce the yield of the PCR.

Primers: In most PCR applications, it is the sequence and the concentration of the primers that determine the overall assay success

Choice of DNA polymerase: Taq DNA Polymerase

Deoxynucleotide triphosphate (dNTP) concentration: Imbalanced dNTP mixtures will reduce Taq DNA Polymerase fidelity.

pH: Generally, the pH of the reaction buffer supplied with the corresponding thermostable DNA polymerase (pH 8.3–9.0) will give optimal results[29.]

Applications of PCR

Cloning, genetic engineering, sequencing[30].

Limitations of PCR and RT-PCR

The PCR reaction starts to generate copies of the target sequence exponentially. The polymerase reaction found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate[31].

DNA profiling is used in the following: In human paternity test, forensic science, Genetic screening, Identifying animal or plants with particular alleles of a gene for selective breeding, Identifying the particular strain of a microbial infection or contamination so that the correct treatment can be applied and the profiling of populations of animals to prevent unnecessary inbreeding in breeding programs and establishing paternity in animals etc.[32].

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

This method of DNA polymorphism analysis was developed independently by two different laboratories[33]. This procedure detects nucleotide sequence polymorphisms in a DNA amplification based assay using only a single primer of arbitrary nucleotide sequence using Polymerase Chain Reaction (PCR). In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template[34]. However, due to the stochastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. They are dominant markers and hence have limitations in their use as markers for

mapping, which can be overcome to some extent by selecting those markers that are linked in coupling[35]. RAPDs are one of the most popular DNA based approaches[36]. RAPDs are the least technically demanding and offer a fast method for providing information from a large number of loci, particularly in species where no study has previously been undertaken.

Moreover, the diversity assessed with RAPDs is comparable with that obtained with other techniques such as isoenzymes[37]. Advantages of RAPDs include suitability for work on anonymous genomes, applicability to work where limited DNA is available, efficiency and low expense[38]. Random amplified polymorphic DNA (RAPD) markers have provided reliable[39] and highly polymorphic information to discriminate pomegranate cultivars[40]. The DNA isolated from an individual is amplified usinga PCR reaction and arbitrary oligonucleotide primers, which will hybrid the complementary sequences, when these exist. The short primers sequences, random paired (8–12 base pairs) are used for DNA RAPD type amplification, usually resulting a presence/absence polymorphism in the gel. A situation like this, which allows the random amplification, in more points, can be realized at the whole genome. Because RAPD is a PCR based method, we will present some important singularities[41]. Polymorphism of RAPD fragments is detected as a band's presence or absence and may result from deletion, insertion or differences in the nucleotide sequence in or between priming regions[42]. Due to these features the RAPD analysis has found many uses in diverse fields of study, such as the assessment of genetic diversity among investigated species or classification of taxa in both plants and animals. The fact that arbitrary primers are potentially capable of amplifying numerous loci in the genome and that the produced DNA fragments have a taxon-specific nature, makes the RAPD technique particularly attractive for the [43] analysis of genetic distance and phylogeny reconstruction[44]. The RAPD analysis has been used extensively in phylogenetic studies of bacteria, fungi, plants, vertebrates and among them– canids. Since[45] first reported on the possibility to apply this technique in dogs, only few more reports concerning the use of the RAPD analysis in canine genetics studies have been published. The RAPD technique to identify polymorphism in canine genomic DNA[46]. Identified RAPD-derived markers specific for the canine Y chromosome. RAPD analysis can potentially detect breed-specific markers.

Such markers have the potential to play an important role in dog identification, or even contribute to developing genetic linkage maps. Furthermore, there is a possibility to develop locus-specific markers (SCAR markers – Sequence-Characterized Amplified Regions) from RAPD markers[47]. PCR amplifications are performed with only one primer composed of SSR units, with or without an anchoring end. ISSR fingerprinting is used widely, especially in plants[48], because of their high level of polymorphism and reproducibility[49] but very little data about such sequences has been published. Photographs of the gels were taken and scoring was done manually. Each amplified fragment was considered as dominant and scored '1', whereas the absence of band was scored as '0' and considered as recessive allele. The generated matrix was analyzed through different softwares to understand the genetic structure. The Nei's average genetic diversity (H) and Shannon index (S)[50] was calculated through POPGENE[51]. The genetic distance (D) and G-test were calculated by TFPGA software with[52] correction. Genetic similarity dendrogram among the specimens were constructed using the Jaccard (J) coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis algorithm in the NTSYS-PC Version 2 computer program[53].

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 modified CTAB method. Isolated DNA was quantified and provided optimized conditions for PCR amplification appropriate reaction mixture using 10 RAPD primers, the PCR product then run on agarose gel electrophoresis, the bands are manually scored from amplification profile on gel. The data obtained by UPGMA method which generate dendogram on the basis of cluster analysis according to Squared Euclidean Distance. Genetic variation and relationship among 4 hibiscus germplasm were analyzed using random amplified polymorphic DNA (RAPD). The phenotypic differences observed in 4 different species using five primers generated 28 bands with size ranging app. 200-3000 bp.On average 5.6 bands generated per primer. Out of the 28 bands, 26 bands (90%) were found to be

polymorphic and 2 bands (10%) were found to be monomorphic.

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