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MOLECULAR PROFILING OF *BAUHINIA* ACCESSIONS USING RAPD MARKERS

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ABSTRACT

The paper is a recent technology of the molecular study dealing with the molecular profiling of species. Here the elucidation of *Bauhinia* accession was done by using RAPD markers. The experiment was done in three steps: (i) Isolation and purification of genomic DNA from leaf tissues of *Bauhinia*, (ii) Generation of molecular profiles of *Bauhinia* using RAPD based DNA marker system and (iii) Evaluation of genetic relatedness of the *Bauhinia* samples under study employing classificatory analysis. To achieve these objectives *Bauhinia* leaf samples were collected from the AAI-DU campus and surrounding areas of Allahabad. These leaf samples were further processed to obtain DNA using CTAB method and SDS method. The isolated DNA samples were separated on agarose gel by electrophoresis and visualized under UV transilluminator. It was observed that the DNAs obtained from CTAB method was of good quality compared to the DNA isolated by the SDS method. Hence the DNA obtained from CTAB method was used for further PCR and RAPD analysis. The RAPD primer screening was done with 15 primers and out of which, the data of 9 primers were used for further analysis. From distance data, NJ tree was generated. The NJ tree indicates that the dendrogram was divided into two groups. The *Bauhinia* samples 8 and 9 were distant from rest of all the Bauhinias whereas the *Bauhinia* samples 11 and 12 were most similar to each other. The present study was done to provide foundation for advanced R&D methodologies such as that ones which employed in Biodiversity and Genetic diversity analysis.

KEYWORDS

Molecular Profiling, DNA, RAPD, markers, *Bauhinia* accessions.

INTRODUCTION

The genus *Bauhinia* is a species of flowering plant in the family Fabaceae, native to South China (which includes Hong Kong) and southeastern Asia. The name is in honour of two botanists John Bauhin and Casper Bauhin who were twin brothers. It is believed that most of the *Bauhinia* species in India were found in Uttar Pradesh and Himalayas, which might be the reason for higher diversity among north Indian cultivars of *Bauhinia* and also for interspersion of species. *B. purpurea* is the most common species to be found in India. The indigenous plant genetic resources are reservoirs of useful genes for crop improvement program. The assessment of diversity has traditionally been carried out through the morphological and cytological characters or horticultural descriptors. However, the efficiency of a selection scheme or

genetic analysis based on phenotype is a function of heritability of a trait. The factors like the environment, multigenic and quantitative inheritance or partial and complete dominance often confound the expression of a genetic trait. However, many of the complications of the phenotype level assay can be mitigated through direct identification of the genotype with a DNA based diagnostic assay using molecular markers. The genus *Bauhinia* has been extensively planted as a garden, park and roadside ornamental tree in many warm temperate and subtropical regions. Along with the ornamental use, leaves, flower buds, young shoots and pods of some of species are also eaten vegetables in the native countries (Baily,1941; Ramasatri and Shenolikar,1974). The pods are eaten either roasted (Namibia) or boiled (Zambia) and also used as a source of vitamin A (Essien and Fetuga,1989).

Many species of *Bauhinia* are used to obtain medicinal products for stomach tumor, cholera, malaria, jaundice, stomach ache, and many more (Jain, 1967). Various species of *Bauhinia* are used as medicinal plants (Jain, 1967); antidote to poison (*B. accuminata*), astringent, treatment of jaundice, small pox (*B. purpurea),* dysentery, dropsy (*B. recemosa),* malaria, tumours, ulcers (*B. variegate*) blood dysentery, loose motion (*B. malabarica*), indigestion, stomach ache (*B. josephii*), bile dysentery, fecal discharge, fever (*B. roxburghii*), ulcer in mouth, bristles on tongue (*B. picta*), cholera, snake bite, sores, (*B. retusa*). Seeds of *Bauhinias* serve as a human food source (Ramasastri and Shenolikar, 1974), as a possible pest control agent (Freedman *et al.,* 1979) and as a source of vitamin A.

Molecular marker is a DNA sequence that is readily detected and the inheritance of the sequence can easily be monitored. These markers are numerous in number and represent a milestone in plant genetics by providing the capacity for complete coverage of nuclear, mitochondrial and chloroplast genomes. The greater utility of molecular markers derives from inherent properties that distinguish them from morphological markers (Tanksley, 1983). These markers show Mendelian inheritance, get stably inherited and remain unaffected by the environment and developmental stages (Liu and Furnier 1993). Saiki *et al*. (1995) introduced polymerase chain reaction (PCR) which led to the further development of DNA based markers. Other markers such as proteins or more specifically allelic variants of several enzymes, also called isoenzymes and other biochemical characters such as lipids or sugars could also be considered (Winter and Kahl, 1995). Many of the plant genotypes are characterized with a DNA based diagnostic assay using molecular markers. DNA markers have enabled characterization of genotypes independent of the influences of environment growth conditions, physiological age of the plant and type of tissue being analyzed (Vainstein, 1993).

The DNA markers closely linked to the traits of interest will have great benefits in crop improvement program (Tanksley *et al*., 1989). The role of DNA markers in crop improvement has been reviewed by many authors (Mohan *et al*., 1997; Joshi *et al*., 1999; Kumar, 1999; Gupta *et al*., 1999; Gupta and Varshney, 2000; Thomas and Raman, 2000; Gupta and Roy, 2002 and Gupta *et al*., 2002). DNA markers have several advantages over traditionally used morphological markers: Numerous DNA markers can be identified in many plant species where morphological markers are limited and they require construction of special genetic materials. Most molecular markers show co-dominant mode of inheritance and can be used to differentiate heterozygote from homozygote whereas dominant/recessive interactions frequently prevent distinguishing all genotypes associated with morphological markers. Molecular markers are generally phenotype neutral, whereas morphological markers frequently cause major alterations in the phenotype, which are often undesirable in breeding populations.

The Molecular marker techniques including RFLP, RAPD, SCAR DAF, ISSR, DAMD, SSR, STMS and AFLP provide modern tools for plant systematics. The DNA markers are used to evaluate the natural polymorphism occurring in

the same genome. These markers are generally classified as hybridization based markers and polymerase chain reaction (PCR) markers. In the hybridization-based markers, the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labeled probe which is a DNA fragment of known/unknown sequence. In case of PCR based markers, the primers of known sequence and length are used to amplify genomic sequences which are visualized by gel electrophoresis technique. The invention of PCR which is a very versatile and extremely sensitive technique uses a thermostable DNA polymerase (Saiki *et al*., 1988; Saiki *et al*., 1995) and has changed the total scenario of molecular biology and has also brought about a multitude of new possibilities in molecular marker research.

As compared to other several PCR based techniques developed during the last two decades, randomly amplified polymorphic DNA (RAPD) technique (Williams *et al*., 1990; Welsh and McClelland, 1990) offers a simple, quick, versatile and economical means of genotype characterization. A discrete DNA product obtained after PCR amplification, if these priming sites are within the amplification range of each other. Welsh and McClelland (1990) produced amplification of several discrete loci introduced this technique with slight variation in primer synthesis or reaction assay or the stringency of the PCR technique. RAPD is a PCR based DNA marker technique, which uses a single arbitrary short oligonucleotide primer (9 - 10bp) instead of a pair of specific primers (Williams *et al*., 1990). The advantages of RAPDs are summarized as the requirement for small amount of template DNA (5-20 ηg), the rapidity to screen for polymorphisms, the efficiency to generate a large number of markers for genomic analysis and the potential automation of the technique (Neale and Sederoff, 1991; Nelson *et al*., 1992; Sobral and Honeycutt, 1993). In addition, no prior knowledge of sequence is required. Since primers can be chosen arbitrarily, any organism can be analyzed with the same set of primers. These advantages make RAPD markers far easier to work and thus very attractive for breeding application (Rafalski *et al*., 1991; Rafalski and Tingey, 1993).

MATERIALS AND METHODS

The chemicals used for preparation of buffers and organic solvents were purchased from Sigma Chemical Company, USA, Sisco Research Laboratories and Merck (India) Ltd. The Agarose was purchased from Bangalore Genei Pvt. Ltd., India and MetaPhor agarose from Genuine Chemical Corporation. For PCR analysis, the different components including *Taq* DNA polymerase and PCR buffer (Bangalore Genei Pvt. Ltd., India), dNTPs (MBI, Fermentas), RAPD primers (Operon Technologies, Inc., USA), 6x loading dye (MBI Fermentas), MilliQ water (Bangalore Genei Pvt. Ltd., India). All chemicals used in the experiments were of Molecular Biology grade. All the glasswares and plasticwares were purchased from Borosil Glasswares Pvt. Ltd., India and Axygen Scientific Inc., USA respectively. The cryogenic container for carrying liquid nitrogen was purchased from Inox (India) Ltd. The important equipments used to carryout this investigation were Thermocycler, Gene Amp 2700 (Applied Biosystems, USA); Agarose Gel Electrophoresis System from Bio Rad; Refrigerated High Speed Centrifuge from Sigma; Deep freezers (-80 and -20), and Water bath from Remi; Lyophilizer and Ice flaking machine from Macro Scientific; Double Distillation Unit of Borosil; UV-VIS Spectrophotometer and Gel documentation system from Spectronics Corporation and Electronic Balance of Denver Instruments. Qualigens autoclavable micropipettes were used for precise micro volumes of liquid handling. Germplasm collection/Leaf samples of all the *Bauhinia* specimens under study were collected from the Allahabad Agricultural Institute-Deemed University campus and the surrounding area viz., Karchana and company garden at Civil Lines, Allahabad, Uttar Pradesh, India and on the basis of collecting samples of unknown accessions they were named as *Bauhinia* sample1 to *Bauhinia* sample12. The genomic DNA was isolated from young, semi-matured leaf samples using Dellaporta (1983) protocol, the isolated

DNA was purified using the standard protocol (Sambrook *et al*., 1989).

DNA Isolation Method (Dellaporta,1983)

i) Two grams of leaf tissue was powered in liquid nitrogen and added in 12 ml of prewarmed 65^0C CTAB buffer (2% w/v CTAB; 0.1M Tris-HCl, pH 8.0, 20mM EDTA pH 8.0, 1.4 M NaCl 1% w/v PVP, Molecular Weight 40000), containing 120 µl 2-mercaptoethanol, till a thick paste was obtained.
ii) The mixture was incubated at 65^0C for 1hour. The slurry was cooled to room temperature. To it 10ml chloroform: isoamylalcohol (24:1 v/v) was added and stirred gently several times to mix the two phases. The mixture was centrifuged at 80000 rpm for 15 min at room temperature.
iii) The aqueous phase was recovered into a fresh sterile tube and the previous step was repeated. The aqueous phase was once again recovered into a fresh sterile tube. To it equal volume of isopropanol and 1ml of 5M Nacl was added and mixed gently by repeated and left at -20^0C overnight.
iv) The mixture was centrifuged at 8000 rpm for 10min at 4^0C. The pellet was twice washed twice with 70% alcohol.
v) The pellet was air dried briefly and rehydrated in 500 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0).

DNA Purification methods (Sambrook et al., 1989)

i) A solution of bovine pancreatic Ribonuclease A (10mg/ml) was prepared in 0.3M sodium acetate, pH 5.0
ii) To each DNA preparation, RNase A was added to a final concentration of 10µg/ml and incubated for 1 hour at 37^0C
iii) The mixture was deprotenized once with Phenol:Chloroform:Iso-amyalcohol (25:24:1, v/v) followed by twice with Chloroform : Iso-amyalcohol (24:1, v/v).
iv) The aqueous phase was recovered and $^1/_{10}^{th}$ the volume of 3 M sodium acetate (pH 5.0) was added to the supernatant. The DNA was precipitated with double the volume of chilled absolute ethanol.
v) The precipitated DNA was recovered by centrifugation at 10000 rpm for 10 minutes at 20^0C and washed with 70% ethanol.
vi) The DNA pellet was lyophilized and re-hydrated with TE buffer (10mM Tris HCl, pH 7.5, 1 mM EDTA, pH 8.0).

Determination of the Quantity and Quality of isolated DNA

The size of genomic DNA isolated is an important criterion for purity. The quantity and quality of DNA was tested by spectrophotometric method at 260 nm and 280 nm, followed by qualitative checking on 0.8 % agarose gel (Manniatis, 1990). The concentration of DNA samples was calculated using the formula given below:

Concentration of DNA (µg/ml) = OD at 260 nm x dilution factor x 50 µg/ml

The ratio of absorbance at 260 to 280 nm indicates the purity of DNA samples. OD of DNA samples should be in the range of 1.7 - 1.8. The presence of impurities like proteins or phenols tend to decrease this ratio and the presence of RNA contamination increases this ratio - i.e., A_{260}/A_{280} = 1.7 - 1.8 will be Pure DNA, A_{260}/A_{280} > 1.9 will have RNA contamination and A_{260}/A_{280} < 1.8 will have Protein contamination. Gel electrophoresis of the genomic DNA was carried out for qualitative analysis of samples prepared. A good DNA preparation appears as a sharp single band. A submarine horizontal agarose slab gel apparatus as described by Sambrook *et al*. (1989) was used. In this technique the open ends of gel tray were sealed with tape and the comb was placed properly. 0.8 g of agarose was dissolved in 100ml 0.5 x TAE buffer in a 250 ml conical flask and was boiled in microwave oven to dissolve the agarose powder completely. Ethidium bromide was added from the stock solution to make a final concentration of 0.5 µg/ml. Ethidium bromide should be added

only after cooling the molten agarose up to 50^0C, it was poured in an electrophoresis tray and allowed to solidify at room temperature. Wells were made by fixing the comb over the tray, prior to pouring the molten agarose. The comb was removed after the solidification of agarose and the gel was transferred to an electrophoresis tank containing 0.5x TAE buffer. DNA of concentration 10ng/µl calculated according to the OD value at 260 nm was taken and mixed with 2 µl of 6x loading dye. The sample DNA was then loaded carefully into well of the casted gel. A DNA ladder was also loaded along with the samples to quantify DNA and electrophoresis was carried out at a constant voltage of 70V. After the dye had run halfway the gel was observed under ultraviolet light and photographed using the gel documentation system. However, the separation of PCR products using agarose gel electrophoresis was carried out with slight changes in the concentration of agarose and in the buffer used.

RAPD analysis

The RAPD reactions were carried out in total volume of 15 µl and contained 4 µl (ca. 50ng template DNA), 0.2 mM each dNTP, 1.2 Units *Taq* DNA polymerase, 2mM Mg^{+2} ion concentration, 10 picomoles primer and 1X Assay Buffer supplied by the enzyme vendor. Here the reaction was carried out at 94^0 C for 2min as a pre-denaturation step, then the reaction was cycled 44 times at 94^0 C for 35second, 35^0 C for 50 seconds and extention at 72^0 C for 1 minutes. Additionally a final cycle allowed extension for 4 minutes at 72^0C. The amplification products in all of the above trials were seprated electrophoretically on 1.0 % agarose gels in 0.5 X TBE buffer, visualized and photographed over a UV transilluminator after staining with ethidium bromide, as described earlier. In order to have a preliminary idea of genetic variability of the *Bauhinia* samples under study, a number of RAPD primers were used. These are random operon primers with 60-80% G+C content and which have designed to have no internal complementary regions. After the screening of primers, for actual data analysis, only those primers were considered which resulted in consistent profiles amongst at least 90% of all the accessions used (Table 1.1).

Table 1.1
List of the primers for which data were scored for RAPD analysis

Primer Name	Primer Sequence(5'-3') / (Length in numbers of bases)
OPA 08	GTGACGTAGG
OPA 10	GTGATCGCAG
OPA 11	CAATCGCCGT
OPB 01	GTTTCGCTCC
OPB 18	CCACAGCAGT
OPC 12	TGTCATCCCC
OPC 20	ACTTCGCCAC
OPF 08	GGGATATCGG
OPF 20	GGTCTAGAGG

The amplified fragments were separated on 1.5% Agarose (Bangalore gene) gel containing Ethidium Bromide (0.5µg per ml) at 70 V for 3-4 hours in Tris Borate EDTA buffer (TBE). After completing the run, the gel was photographed under UV excitation using Spectroline Bio-Vision Gel Doc System. All the PCR reactions were repeated at least twice to check the reproducibility. Each PCR was conducted as an experiment, with controls (distilled water

instead of template DNA) to test the purity and viability of reagents (Elrich *et al*., 1991).
Data analysis was done for only those genotypes that resulted in giving reproducible and consistent profiles. In order to describe the genetic diversity among the different species of the genus *Bauhinia*, RAPD band data were used to estimate genetic distances, based on Jaccard's similarity coefficient (Jaccard, 1908) using the Free Tree Program. Cluster analysis was carried out based on genetic distances using Neighbour Joining Program (NJ) in the Free Tree package.

RESULTS AND DISCUSSIONS

The work on molecular profiling of Bauhinias and their characterization was initiated with the isolation of DNA from the leaf tissue collected. The DNA isolation protocol were followed viz., Dellaporta (1983) using leaf tissues. The quality of genomic DNA of isolated samples was checked by electrophoresis on 0.8% agarose gel. The DNA band migrated equivalent to lower than 23.1 kb band of EcoRI double digested λ DNA. The *Bauhinia* DNA samples isolated by both the methods were electrophoresed separately on agarose gel and visualized under UV transilluminator. The protocol yielded DNA with good quality and quantity of isolated DNA as shown in Figure 1.2

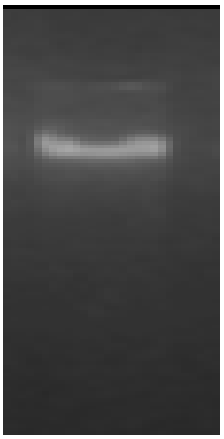

Figure 1.2: ***DNA isolated from Bauhinia leaf tissues by using Dellaporta (1983) Protocol.***

The DNA isolation protocol of Dellaporta (1983) was employed to isolate genomic DNA from all 12 samples and they exhibited the A_{260} / A_{280} ratio between 1.7 to 1.8 after purification. Based on the spectrophotomertic readings, the DNA samples were diluted to 12.5ηg/µl and used for RAPD analysis. The same result and their applications was obtained by other eminent research workers (Katterman and Shattuck, 1983; Peterson *et al*., 1997 and Porebski *et al*., 1997).
It was observed that from the gel that DNA isolated by the CTAB method is superior in quality and quantity. Further, the isolated DNA was tested in pilot PCR reactions and screened with 15 primers and here also, the DNA was found to be of a quality good enough for the rest of the study. The RAPD analysis was carried out with the primers that seemed to have given good profiles during the initial screening. Nine such primers viz., OPA 08, OPA 10, OPA 11, OPB 01, OPB 18, OPC 12, OPC 20, OPF 08 and OPF 20 generated good profiles consisting of distinct well-separated bands on the agarose gels. However, it was observed that one of the DNAs (*Bauhinia* Sample No.10) was inconsistent and did not give any profile with some of the primers tested. For all other DNAs multi-banded profiles were observed. Typically RAPD profiles in case of six of the primers analyzed viz., OPA 08, OPA 11, OPB 01, OPB

18, OPF 08 and OPF 20 showed consistent polymorphic banding patterns. All the bands scored from the nine primers were considered cumulatively to determine the pairwise distances amongst the Bauhinias according to Jacard (1908) using the program Freetree. From the distance data, a 500 replicate bootstrap analysis was carried out and a NJ tree generated using the same program. The present study on the genetic variability within and among *Bauhinia* accessions collected were efficiently carried out employing RAPD based DNA markers. Preliminary screening facilitated the selection of primers producing a higher level of polymorphism and more reproducible fragment patterns. Once identified, the selected primers could also serve to evaluate a large number of genotypes of *Bauhinia* in future analysis (Carol *et al*., 2005).

The work on Bauhinias was initiated with 12 trees selected from amongst the ones growing around Allahabad. These 12 Bauhinias included mainly 2 groups and second group was again subdivided. The twelve DNAs for which discrete profiles were scored in case of the nine RAPD primers generated some useful data and information. In the dendrogram, all the 12 Bauhinias were divided into two groups i.e., group1 and group 2 at 100 units in the dendrogram. Group 1 includes *Bauhinia* sample No 8 and *Bauhinia* sample No 9. The DNAs of *Bauhinia* sample No 8 and *Bauhinia* sample No 9 were considerably different from that of the other Bauhinias. The group 2 includes rest of the 10 Bauhinias. It ranges lowest from 9 units to the highest of 100 units. Group 2 was further divided into two subgroups 2.1 and 2.2. Subgroup 2.1 was further subdivided at 45 units of the dendrogram. It includes *Bauhinia* sample No 1, *Bauhinia* sample No 2 and *Bauhinia* sample No 3. *Bauhinia* sample No 1 and *Bauhinia* sample No 2 were divided at 96 units of the dendrogram. Subgroup 2.2 was divided at 9 units of the dendrogram, 7 Bauhinias came in this cluster, *Bauhinia* sample No 4 was distant from them. Again at 93 units, *Bauhinia* sample No 10 separated from rest of the 5 Bauhinias and these 5 Bauhinias were further subdivided into two, at 72 units of dendrogram. *Bauhinia* sample No 11 and *Bauhinia* sample No 12 were similar to each other. It was clearly indicated that the *Bauhinia* sample No 5 was similar to *Bauhinia* sample Nos 6 and 7 whereas these samples (6 and 7) were distant from each other. Two other Bauhinias, *Bauhinia* sample No 8 and *Bauhinia* sample No 9 were most distant from all other Bauhinias.

The study though very preliminary one, has nevertheless resulted in some interesting observations. The Bauhinias are a good subject of study and show clear level of demarcation. This is important because in many parts of the country, the Bauhinias have been observed to be growing so prolifically that these have almost been considered as weeds. A major R&D effort into this interesting genus of ornamentally and medicinally important plants is thus justified since the variability exhibited at the morphological level is also revealed upon DNA analysis of different genotypes.

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