Computer Application of Ward's method for RAPD analysis of mulberry varieties

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Abstract

Many statistical techniques that allow finding similarities or differences among data and variables. Cluster analysis encompasses many diverse techniques for discovering structure within complex sets of data. The objective of cluster analysis is to group either the data or the variables into clusters such that the elements within a cluster have a high degree of "natural association" among themselves while clusters are "relatively distinct" from one another. To do so, many criteria have been described: partitioning methods, arbitrary origin methods, mutual similarity procedures and hierarchical clustering techniques. One of the most widespread hierarchical clustering methods is the Ward's method.

The present investigation revealed that the 19 mulberry plants are dioceious and cross pollinated with each other to produce fertile hybrid revealed a closer genetic relationship.This cannot be noticed at the species level.Usually traditional methods like morphological characters/traits are not very successful in establishing the diversity and relationship among different mulberry varieties because of environment influence. PCR based molecular marker method; RAPD was employed to study the genetic diversity and inter-relationships among 19 mulberry varieties. On an average, RAPD analysis generated 43 discrete bands/varieties with 5 arbitrary primers. The size of the amplified products ranged from 300-5000 bp with an average of 2-4 bands per primer. Of 49 amplified fragments, 41 were polymorphic (94%) with at least one pair-wise comparison between 19

varieties. RAPD analysis identified varieties specific amplification products, which will be useful in germplasm classification and introgression studies. These results suggest that RAPD based markers are useful for genetic characterization of mulberry speices/ varieties.

Keywords: Mulberry, RAPD Regionalized variables; generalized Ward's method;

Introduction

There exsists considerable difference of opinion in classification of species of Morus by several Systematics based on morpholigcal characteristics (Linneaus, 1753; Koidzumi,1917 and 1923; Hotta1958; Katsumata, 1972 and Airyshaw, 1973). Traditional methods of genetic analysis based on morphological characteristics are time consuming and error-prone due to environmental variation (Wang and Tanksley, 1989). Genetic analysis of various characters is relatively difficult in mulberry due to its prolonged reproductive cycle and also the dioecious nature which prevents selfing.

Genetic resource available for varietals improvement are abundant within the mulberry species. In India , two eco species i.e, M.laevigata and M.serrata have been generally recognized (Hooker, 1885) and their genetic differentiation has been investigated by morphological , anatomical, reproductive and isozyme and analyses.

The markers based on isozymes have been used extensively to characterize the genetic resource (Jana and Pietzark, 1988; Chengyin et al., 1992) and to study the phylogenetic relationships in Mulberry varieties (Hirano, 1980). Isozymes or protein variants require separation by electrophoresis, and are visualized by enzyme activity assay for the relevant enzyme. They are generally selectively neutral, well disturbed over the genome and are co-dominant. The variants of these often do not have any noticeable effect on phenotype, thus making them suitable markers. However, the number of genetic markers provided by

the isozyme analysis is barley sufficient for many applications in crop improvement programme.

The advent of recombinant DNA technology heralded a completely new approach to define potentially polymorphic DNA sequences. This new technology promises to revolutionize some areas of plant genetics and plant breeding. The markers based on DNA sequences have introduced a new dimension to the development of genetic maps and mapping of agronomically and physiologically important characters.

The major strength of DNA markers is that they have a potential to reveal almost unlimited number of polymorphisms covering the whole genome. The DNA markers have very unique feature like ubiquitous nature, detection at any developmental stage and independent of environmental effects and management practices, and hence have direct applicability to breeding programmes. The most important application of DNA marker technology is the generation of saturated linkage maps which have been extremely useful for mapping and tagging of genes of agronomically important traits.

RFLP's (Restriction Fragment Length Polymorphisms) are the first of the DNA markers which have been widely used for construction of genetic maps and assessment of genetic variability .RFLP's have made it possible to generate high density maps of various commercially important crops like rice, Tomato, Lettuce and Soybean.

The development of the polymerase Chain Reaction (PCR) for amplifying DNA led to a revolution in the applicability of molecular methods and a rangeof new technologies were developed which can overcome many of the technical limitation of RFLP's such as

developing probes for RFLP analysis, Southern Hybridization and use of radioactive isotopes, which render them unsuitable for large scale genomic studies. A subset of the latter involves the uses of a single 'Arbitrary' primer which results in amplification of several discrete DNA products. The method is referred to as Random Amplifed Polymorphic DNA (RAPD) analysis.Genetic analysis with RAPD marker is more rapid and simpler than RFLP analysis and requires smaller amounts of DNA. Moreover, it does not require cloning of DNA or Southern hybridization. However, unlike RFLP's, most of the RAPD markers are usually scored as dominant and heterozygotes cannot be identified from homozygotes.

RAPDs have been demonstrated to be well suited for use in plant and animal breeding programme, population genetics and for estimating genetic diversity and relatedness in plant populations or cultivars (Halward et al.,1992; Jain et al., 1994; Yu and Nguyen 1994; He et al., 1994)

In India, there are hundereds of collections of diverse mulberry accessions whose genotypic status is not known. Beside, an in depth genetic analysis of mulberry collections has not been carried out so far. By making use of the molecular marker technology based on PCR approach, diverse accessions of Mulberry.

Materials and metohds:

The present investigation was carried out in the Department of life science, Bangalore university, Bangalore during the year January-2013 to April-2014.The materials used and methods followed in this study are presented here.

The present study selected leaf samples of 14 mulberry varieties plants that were collected from Bangalore University, Mulberry Germplasam. Which may represent the wide

variation prevalent in the genome. The recently matured leaves were collected and used for DNA extraction.

The basic DNA extraction protocol Dellaporta et al. (1983), was slightly modified following Porebski et al., (1997). 1g of fresh leaf sample of each plant was grinded using liquid nitrogen and then transferred to a centrifuge tube containing 20 ml of extraction buffer (3% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP and 1% B-mercaptoethanol) preheated to 60º C and maintained at this temperature for 1 hour with intermittent shaking. The centrifuge tube was brought to room temperature and 6 ml of chloroform and isoamyl alcohol (24:1) were added. The contents mixed well by inverting the tube gently 25-30 times, then spun at 7,000 rpm for 15 min. The supernatant was transferred to centrifuge tube and this clean-up step was repeated until a clear supernatant was obtained. Supernatant was kept overnight at 4º C to precipitate DNA by adding half a volume of 5 M NaCl and one volume of isopropanol. The DNA was pelleted by centrifuging at 10,000 rpm for 20 min and the pellet was washed with 70% ethanol. The dried DNA pellet was resuspended in 300 µl of TE (Tris EDTA) buffer. Contaminating RNA was removed by digestion with 10 µg of Rnase for 60 min at 37º C. Proteins were removed by digestion with 25µg of Proteinase-K. The DNA was further purified by extracting twice with an equal volume of phenol followed by an equal volume of phenol: Chloroform (1:1) and finally with an equal volume of chloroform. The DNA was precipitated by the addition of one volume of isopropanol and spun at 5,000 rpm for 5 min; the final pellet was dissolved in 300 µl TE. The DNA concentration was determined using UV-Visible spectrophotometer at 260 nm and 280nm and the quality verified by electrophoresis on a 0.8% agarose gel and diluted to a uniform concentration of 5 ng/µl for RAPD analysis.

DNA electrophoresis

Amplified DNA fragments were separated out on 1.2% agarose gel stained with ethidium bromide. Running buffer containing Tris-buffer, Acetic acid and EDTA (pH 8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 25µl reaction volume and 5 µl of loading buffer (Sucrose, Bromophenol blue and Xylene cyanol) together. Electrophoresis was conducted at 45 volts for 3 hours and the gel photographed under UV light using gel dock system (Herolab).

Statistical analysis.

Binary coding was used to score gel and each band of primer was scored of 6 species and 12 primers with 100 to 1000 base pairs Marker level pair wise squared Euclidean species was calculated and utilizing these distances, species were clustered following Wards method. The segregation of species was also assessed through principle component analysis, the Statistica version 5.0 a computer application was used to generate dendrogram using squared Euclidean distance and Wards method.

Quantification of DNA

DNA quantification can be done by flurometry, spectrometry and agarose gel electrophoresis with standard DNA concentrations (Boiteux *et al.,* 1999). The quality can be assessed by restriction digestion with restriction endonucleases (EcoRI, HindIII etc.,) electroporation and spectral properties. Quality is that to what extent the DNA is pure of secondary metabolites and other substances, which hinder further use of DNA in molecular techniques. A good DNA preparation generally exhibits the following spectral properties. It will have A_{230}, A_{230}/A_{260}, A_{280}/A_{260} or A_{260}/A_{280} rations of less than 0.10, less than 0.45, less than 1.65 or more than 1.80, respectively (Shantha *et al.,* 1998). If a DNA preparation exhibits A_{260}/A_{280} more than 1.80, it shows the presence of RNA and if it is less than 1.65 or less indicates protein contamination (Sambrook *et al.,* 1989).

Results

Extraction of genomic DNA

The genomic DNA was extracted as described in Materials and Methods from fresh and young leaves of mulberry varieties. The extraction method yielded a good amount of DNA on test gels. It gave a good resolution (Fig. 1). The quantity of DNA/gm of fresh leaf tissue and relative ratio of DNA to proteins are given in Table 1.

Purification of DNA

Since the DNA samples using the Nucleon Phytopore method contained a considerable amount of RNA, polysaccharides and other impurities, it was necessary to go for further purification to get a good quality DNA essential for RAPD analysis.

Optimization of amplification conditions for mulberry DNA

The mulberry DNA was amplified with slight modifications according to the protocol of Williams (1990). An initial denaturation of 2 min resulted in better separation of DNA strands allowing binding of random 10-mers to template DNA for sequence-specific PCR amplification. Amplification of mulberry genomic DNA with random oligonucleotide primers resulted in a series of discrete bands of varying intensity.

Table 1. DNA content of the samples and relative ratios of DNA to protein .

Varieties	260 nm (A1)	280 nm (A2)	A1/A2	DNA content (µg/µl) x 300 µl/gm fresh leaf tissue
1	0.079	0.044	1.8128	0.395
2	0.047	0.031	1.5079	0.470
3	0.049	0.033	1.5037	0.490
4	0.039	0.026	1.5023	0.390
5	0.068	0.046	1.4773	0.340
6	0.065	0.049	1.3366	0.325
7	0.127	0.078	1.6304	0.635
8	0.063	0.044	1.4478	0.315
9	0.067	0.045	1.4809	0.335
10	0.021	0.019	1.0816	0.105
11	0.071	0.047	1.5129	0.355
12	0.072	0.047	1.5306	0.360
13	0.046	0.035	1.3054	0.230
14	0.032	0.025	1.2628	0.160
15	0.049	0.033	1.5037	0.490
16	0.039	0.026	1.5023	0.390
17	0.068	0.046	1.4773	0.340
18	0.065	0.049	1.3366	0.325
19	0.127	0.078	1.6304	0.635

Taq DNA polymerase concentration

The most important parameter required for consistent amplification patterns was found to be concentration of *Taq* DNA polymerase. Concentrations of 0.2 to 1.0 U of *Taq* DNA polymerase per 20 u.1 reaction mixture were used for RAPD-PCR reactions. 0.3 U and 0.4

U of *Taq* DNA polymerase in a 20 (J.I reaction volume was found to generate discrete bands (Fig.1). At optimum *Taq* DNA polymerase concentration of 0.3 U/20 u.1 reaction volume, the RAPD profile remained consistent and reproducible.

RAPD analysis

A total of five decamer oligonucleotide primers were examined on the 19 mulberry varieties.All the random primers resulted in distinct polymorphic banding pattern. Results obtained with the primers OPW-01, OPW-02, OPW-03, OPW-04 and OPW-05 are shown in Fig.1.The number and size of the amplification products varied depending on the sequence of random primers and mulberry varieties.The size of the amplified products ranged from 300-5000 bp with an average of 2-4 bands per primer. A total of 43 discrete amplified products were generated by 5 random primers. Of 43 amplified products, 41 (94%) fragments were polymorphic in a pair-wise comparison and the remaining 2 fragments were monomorphic across the accessions investigated. Table 1 and 2 gives a summary of the RAPD patterns.

Table 1. Summary of the detection of RAPD markers

Total number of primers	5
Total number of bands amplified	43
Size range of amplification products	0.3 to 5.0 kb
Average number of bands as primer	2-4
Total number of Polymorphic bands (RAPDs) identified	41
Percentage of total bands which were polymorphic	94%

Table2. List of Primers which gave polymorphic / monomorphic bands (RAPD)

Primer Code	Total Number of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands
OPW-01	8	8	-
OPW-02	7	7	-
OPW-03	6	5	1
OPW-04	10	9	1
OPW-05	10	8	-

Fig.1.Gel Diagram of 19 mulberry varieties

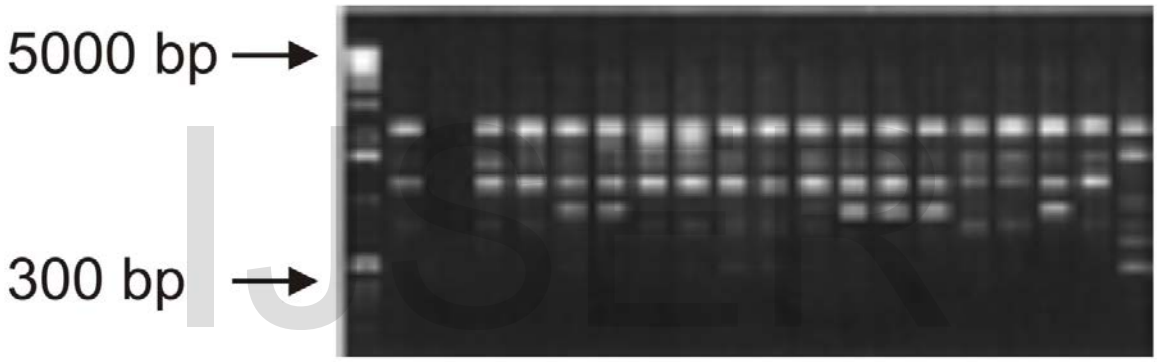
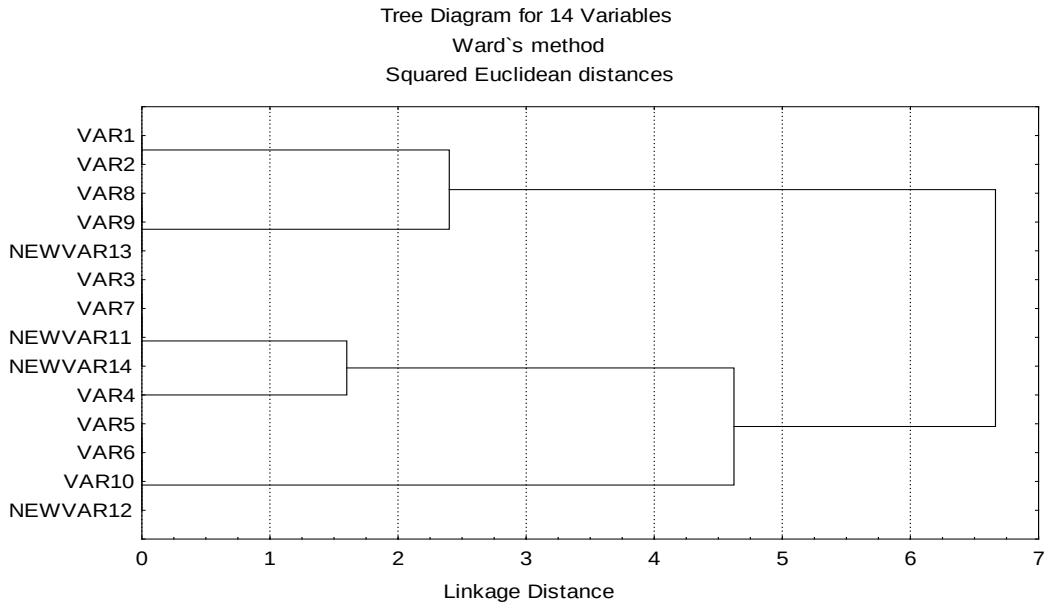


Fig 2 : S13, S146, S34, V11, M5, Mysore local, R-175, DD, Srinagar local, MR2, S1635, Karanahalli local, S36, *M. macroura, M.rotundiloba,* Chinapeaking, *M. Ihonseringe,* S11, Assambola, S41



Note variable for 15,16,17, 18 and 19 is not shown

Fig 2 : S13, S146, S34, V11, M5, Mysore local, R-175, DD, Srinagar local, MR2, S1635, Karanahalli local, S36, *M. macroura, M.rotundiloba,* Chinapeaking, *M. Ihonseringe,* S11, Assambola, S41

Discussion:-

The mulberry species are good genetic resources, some of the species of mulberry have reported naturally in India and Japan. Some of the cultivate species / varieties do not find-their origin outside the country specially 'Chaina & Japan'. All most all cultivated varities which are classified as different species. The mulberry species were cross pollinate with each other and produce pertile off spring indicating no signs of sexual incompatibility characeteristics of the species. This information suggests a closer genetic relatinship among cultivated mulberry vrieties. The present investigation was carriedout on 14 mulberry varieties and its RAPD band analysis further support in this direction.

The high level of polymorphism (94%) in mulberry accessions reflects the out crossing nature on par with results obtained with RAPD in other fruits and nut tree species such as

pistachio (Hormaza, 1995), olive (Fabbri *et al.*, 1995), walnut (Nieise *et al.*, 1998) and vegetatively propogated crops such as banana (Krammer *et al.*, 1992) and apple (Koller *et al.*, 1993).The molecular analysis using RAPD markers did not show correlation between the ploidy and number of polymorphic bands suggesting the redundancy of genome complements in these polyploid species. However, the ploidy level of a plant does not appear to influence the number of fragments per primer (Wolf and PetersVanRijn, 1992). In mulberry accessions percentage of similarity based on the F-value ranged from 97.3 to 41.4. Most of the mulberry pairs had high similarity value. Even though some morphological variability and improvement in agronomic traits, leaf yield have been achieved through selective cultivation and breeding programmes, there appears to be no effort has been made to utilise the useful traits found in wild mulberry species.

As reported by earlier literature, many cultivated mulberry varieties find their origin elsewhere; which are later adapted, improved consciously or unconsciously in different regions of the country. Many such accessions do not carry clear pedigree record nor an authentic information on their origin. In this background, expectedly RAPD profiles of 10 mulberry varities showed 2-3 RAPD bands show any correlation with regional/geographical distribution. The varities like 2 and 9 forms a single cluster whereas varities like 8 and 5 forms second cluster,variety 11 and 4 shows third cluster and varieties 19 and 10 revealed fourth cluster despite the varieties 1 and 12 are not related to any other varieties as shown in dendrogram fig.2.These accessions were evolved from the breeding generation of Mulberry accession (2n). As per the earlier report The popular accession of Southern-India namely, Kanva 2 is believed to be OPH selection from the traditional Mysore Local. Tl which is a colchiploid (4X) was evolved from the cross

between *M. indica* and Mandalaya (Sl) has a similarity of 64.9% with the female and 86.5% with the male parent.

However similar investigation was carried out on Matigara White, TR-8 and T-l which are diploid, triploid and tetraploid respectively fall in the same cluster indicating the common geographic origin i.e., and also shared common morphological characters such as homophyllous nature and ovate shape of the leaf and pubescent nature of the stigma. *M.rotundiloba and* Chinapeaking also fall in same cluster sharing similiar morphological characters such as homophyllous nature, green colour, smooth surface and ovate shape of the leaf, although originating in different countries. S13, S146, S34 originating from *M. macroura* also fall under the same cluster along with Punjab local and Himachal local (Swarna Latha 2000).

The varietal specific RAPD bands may be used as a probe to screen mulberry genome and ascertain whether they are low/high copy number in the genome and such specific bands may be converted as sequenced characterised amplified regions (SCARs) by sequencing the amplified products and designing a pair of primers. The present study though preliminary, has supported many of the earlier findings. More information on the genetic background to be generated before arriving at concrete conclusion. However, higher resolution of this PCR based technique in mulberry in contrast with low level of polymorphism of isozymes and proteins has proved to be a valuable tool for generation of genetic information to identify and assess the genetic diversity.

The present study reveals that RAPD markers are good choice for assessing the genetic diversity and relationship in 19 mulberry varieties with polymorphism levels enough to

establish informative fingerprints with a few markers. The information obtained could be of practical use for mapping the mulberry genome as well as for classical breeding. The study also provides a closer basis for mulberry breeders to make informative choices on selection of parental materials based on genetic diversity and overcome the problem usually associated with tree crop improvement programme. The highly informative primers identified will be useful in molecular characterization of gene bank acessions.

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