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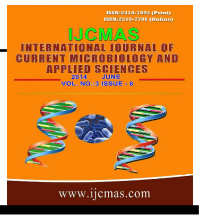


**Original Research Article**

# An investigation of RAPD and ISSR molecular marker studies in Mulberry varieties

**B.K.Chikkaswamy* and Rabin Chandra paramanik**

Department of Biotechnology, Sigma BioScience, Research Center, 2nd stage, Indiranagar, Bangalore, India
**Corresponding author*

**A B S T R A C T**

Mulberry (*Morus spp)* plants are dioecious in nature, reproductive cycle and prone to morphological variation within the species due to environmental impacts. Due to these reasons it is difficult to differentiate between the varieties/cultivars of Morus spp. The present study was undertaken to find the degree of polymorphism within the varieties using Random Amplified Polymorphic DNA (RAPD) technique. 18 cultivars of mulberry were evaluated for variability using a set of 16 random 10 mer oligonucleotide primers and 4 SSR primers. The number of markers generated per primer ranged from 0 to 6. 61 RAPD and 43 SSR bands which are polymorphic in nature were formed. The genetic distances between each of the varieties were analyzed by generating dendrogram, which shows phylogenetic relationships between them.

**K e y w o r d s**

Mulberry, Polymorphism, RAPD, SSR, Phylogenetic relationships.

## Introduction

There exists a considerable difference of opinion in the classification of species of M*orus* by several systematics based on morphological characteristics (Linnaeus, 1753; Koidzumi, 1917 and 1923; Hotta, 1958; Katsumata, 1972 and Airyshaw, 1973). Traditional methods of genetic analysis based on morphological characteristics are time consuming and error-prone due to environmental variations (Wang and Tanksley, 1989).

Amplification of genomic DNA using a random sequence oligonucleotide primers became very handy and is being widely used although it was discovered only recently (Williams *et al.*, 1990; Welsh and McClelland, 1990). Random Amplified Polymorphic DNA (RAPD) is useful method where little or no sequence data is available for extensive background research. Genetic analysis with RAPD and SSR markers is more rapid and simpler than RFLP analysis and requires smaller amounts of DNA.

Simple Sequence Repeats (SSR) are a technique which utilizes primers based on microsatellites to amplify inter-SSR DNA sequences on SSR (Zietjiewicz *et al* 1994) Here, various micro satellites anchored at the 3' end is used for amplify genomic

DNA which increases their specificity. These are mostly dominant markers, though occasionally a few of them exhibit co-dominance. An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and pentanucleotides, *etc.* with an anchor made up of a few bases and can be exploited for a broad range of applications in plant species (Souframanien and Gopalakrishna., 2004; A.B. Danger *et* al., 2006; Basha and Sujatha 2007).

## Materials and Methods

Leaf samples of 18 mulberry species/varieties namely MOULAI , PAPU NEW GUNEIA , LONAWALE, RFS-135, C-20, CHINA WHITE, C-1724, T- 10, MI-0556, T-8, T-12, MS-5, SRDC-1, M. ALBA L., C-763, BIRDS FOOT, S-13, ZIMBABWE-1 were collected from the germplasm maintained by the Department of Sericulture, Bangalore University, and Bangalore. The first 2-4 leaves from the tips were collected and used for DNA extraction.

Isolation and purification of total genomic DNA were carried out according to the protocol suggested by Porebski *et al*., (1997) with slight modifications. 2g of fresh leaf tissue was grinded using liquid nitrogen and then transferred to a tube containing 12 ml of extraction buffer (3% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% PVP and 1% β-mercaptoethanol) preheated to 65°C and maintained at this temperature for 1 hour with intermittent shaking. The centrifuge tube was brought to room temperature, spun at 7,000 rpm for 15 min at 4°C and collected the supernatant to which 6 ml of chloroform and iso-amyl alcohol (24:1) were added. The contents were mixed well by inverting the tube gently 25-30 times, and then spun at 7,000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh tube and repeated the same step thrice. Supernatant was kept overnight at 4°C to precipitate DNA by adding half a volume of 5 M NaCl and equal volume of iso-propanol. The DNA was pelleted by centrifuging at 12,000 rpm for 20 min and the pellet was washed with 70% ethanol. The dried DNA pellet was resuspended in 1ml TE buffer. 20µl RNase was added and incubated for 60 min at 37°C. The DNA was further purified by treatment 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 iso-propanol and spun at 12,000 rpm for 20 min at 4°C. Finally 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.

### RAPD

The basic protocol reported by Williams *et al.,* (1990) for PCR was followed with slight modifications. Amplification reactions were carried out in 25 µl reaction mixture containing template DNA (30 ng), 10 pmol of primer ( Operon technologies USA, Inc.), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 U *Taq* DNA polymerase (Hi-media) and 200 µM of each dNTPs (Hi-media).

### SSR

PCR amplification was carried out according to Zietkiewicz *et al.* 1994.SSR analysis of genomic DNA of 45 accessions was carried out using 8 selected ISSR primers (UBC- 807, 809, 810, 811, 812, 820, 825, 828), obtained from the

University of Columbia, Vancouver, Canada.. The reaction was set up in a total volume of 20 µl in a 0.2 ml PCR tube as described below:

30ng Template DNA, 30 pmol Primer, 100 µM dNTPs, 0.5U *Taq* DNA polymerase(Hi-Media), 10mM Tris HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$. The amplification reaction was carried on Corbett Research PCR machine (CG1-96). The thermal profiles used was : Initial denaturation of 94ºC for 5min, 45 cycles of Denaturation at 94 ºC for 1 min , Annealing at Tm for 1 min, Extension at 72ºC for 2 min and Final extension at 72ºC for 5 min. The PCR amplified samples were analyzed on a 1.5% Agarose gel.
Binary coding was used to score gel and each band of primer was scored of 20 varieties and 9 primers with 100 to 1000 base pairs marker level pair wise. Squared Euclidean distance was calculated and utilizing these distances, species were clustered following Ward's method. The Statistica version 5.0 a computer application was used to generate dendrogram using squared Euclidean distance and Ward's method.

## Results and Discussion

The selected 16 primers were used for the screening of varieties / accessions of mulberry (OPA-01, OPA-03, OPA-13, OPA-18, OPB-17, OPC-01, OPC-02, OPC-08, OPC-10, OPC-12, OPD-11, OPD-13, OPF-17, OPE-07, OPE-19 and OPF -07), which generated 70 distinct markers. Both monomorphic and polymorphic bands were considered for the precise calculation of genetic diversity. Fig.1and 2 shows a representative DNA fingerprint generated by the RAPD and SSR primers-809, UBC-807, UBC-810, and UBC-811, table-1,2 and 3 gives the list of random primers used for the present study. A dendrogram based on Ward's and UPGMA method of analysis (Fig. 2,).

### DNA polymorphism among the genotypes

On the basis of the DNA markers generated by the primers, considerable genetic diversity was observed among the varieties. The bands profiles generated by the 16 RAPD primers out which OPD-11 gave 75 RAPD bands, at which 61bands are polymorphic and 14 bands are monomorphic revealed clear variability (Fig. 1). These bands are laid in between 300 – 5000bp with an average of 4.6 bands per primer with the primer sequence AGCGCCATTG. The number and size of the amplification products varied depending on the sequence of random primers and mulberry accessions.

The primers resulted in distinct both monomorphic and polymorphic banding patterns. Further, the isolation and the gel profiling system showing variation in amplification because of the primer sequence. The average number of bands number of bands per primers is 4.6 and the percentage of polymorphic was 81.3%.

Genetic similarity among the 18 mulberry varieties were estimated on the basis of the SSR banding profiles generated by each primer with the genomic DNA of the mulberry varieties. The genomic DNA of these mulberry varieties was amplified with oligonucleotide primer UBC - 809 generated a total number of 43 SSR bands. The number of SSR is specific to each mulberry varieties are showing differential distribution on gel profile. The size of the amplified product ranged from 0.5-1.0kb. Out of these 43 bands, four bands were recorded in varieties such as 10, 16, 17, 18 respectively. However, varieties such as 5, 4, 9, 11, 14, 18, recorded three bands

each respectively. Even though the genomic DNA of mulberry varieties such as 2, 12, 18 amplified with primer UBC – 809 revealed two bands only. It was also noted that only one band was recorded in variety 6. The data showed very high SSR banding pattern to distinguish in mulberry varieties as a diverse character the identification of SSR banding pattern is important because it follows Mendelian inheritance character, despite, some the varieties such showed thin SSR banding patterns on gel profile.

Despite some of the genomic DNA of mulberry varieties revealed less SSR banding pattern in varieties such as 2,6,7,12,14, The data clearly indicate diverse distribution of SSR banding pattern on gel profile, therefore it is useful to correct mulberry identity to one another as an additional tool to support RAPD molecular system as shown in Fig.2. Out of 4 SSR primers UBC-809, 807, 10 and 11 clearly revealed such variability among the genotypes **(**Fig. 2**).** The 4 SSR primers generated a total of 43 bands, of which 42 were polymorphic. Generating 97.6 % polymorphism among the 18 mulberry genotypes.

The dendrograms realized from the above matrices, RAPD and SSR, and the pooled data from both markers using Wards methods of Euclidean distance and UPGMA method grouped the 18 mulberry genotypes into three, clusters (Fig. 2,). The dendrogram of the both RAPD and SSR data revealed genetic diversity and relationships of eighteen mulberry varieties using the UPGMA method. The dendrogram of RAPD of eighteen mulberry showed three clusters. The cluster one included varieties such as MOULAI , PAPU NEW GUNEIA , LONAWALE S-13, ZIMBABWE-1 M. ALBA L., C-763, BIRDS FOOT which is not related with each other, where as varieties Moulai showed a relationship with Papu New. The variety Zimbabwe-1 showed a relationship with S13; where as other varieties are diversified with one another (Fig.2). The varieties papu new. Showed relationship with Moulai whereas other varieties revealed diversity between each other (Fig. 2).

The dendrogram of SSR data 18 mulberry varieties revealed that China white, C-1724, T-18, T-10, MI-0556, M5 and SRDC-1are related with each other (Fig.2). In dendrogram The RAPD and SSR data revealed that .*RFS-135, C-20 and T-12* are interrelated with other ( Fig.2). Futher revealed that all the varieties interrelated with other except few varieties.

The dendrogram of the both RAPD and SSR data revealed genetic diversity and relationships of eighteen mulberry varieties using the UPGMA method. The dendrogram of RAPD of eighteen mulberry showed three clusters. The cluster one included varieties such as Moulai, Papu new guneia, Lonawale S-13, Zimbabwe-1 M. alba l., C-763, Birds foot, which are related with each other, where as varieties *China white,* showed relationship with C-1724. The variety T-8 showed a relationship with T-10, whereas other varieties are diversified with one another (Fig. 2). The dendrogram based on RAPD data revealed that MI-0556, M5 and SRDC-1 are related with each other ( Fig. 2). Similar Observation was made on ISSR data (Fig. 3,).

The present investigations clearly demonstrate the usefulness of RAPD and SSR to delineate the interrelationships among varieties/ genotypes of 18 mulberry varieties present in Karnataka India.

Although SSR primers unraveled more polymorphism than RAPD primers, both RAPD and SSR primers generated almost similar types of genetic relationships among the genotypes and their respective varieties. Using SSR primers, high genetic variability has been detected among closely related cultivars and (or) varieties in many other crop plants (Tsumura *et al*. 1996, Bornet *et al*. 2002). Similarly, Vijayan and Chatterjee (2003) also obtained high genetic divergence among 11 closely related local cultivars of mulberry with SSR primers. Likewise, Bhattacharya and Ranade (2001) and Chatterjee et al. (2004) demonstrated the suitability of RAPD primers in unraveling the genetic relationships among a few genotypes of mulberry indigenous to India. However, there was no report where molecular markers were used to address the problems pertaining to taxonomic identifications in mulberry.

Therefore, this is the first attempt in this direction and the data presented in this report suggest the possibility of using DNA markers to resolve some of the problems associated with the taxonomical classification in mulberry. Furthermore, in this investigation, we have used three different algorithms to estimate the genetic similarities among the genotypes and their corresponding varieties. All three types of matrix and their corresponding dendrograms showed more or less similar results. However, from the dendrograms it is clear that the coefficients of Nei and Li (1979) generated trees with deep and distinct nodes. Hence, coefficients of Nei and Li (1979) could be of much use in the mulberry for phylogenetic studies.

Regarding the genetic relationships of varieties / genotypes, the pairwise estimation of genetic similarity coefficients and subsequent clustering of the genotypes revealed close genetic similarity among the varieties / genotypes of *Morus.* The grouping of genotypes of *S*-13 and T-12 is a separate cluster indicated its greater genetic divergence from other species/ varieties, further, analyses with average genetic distances among the genotypes under each species also revealed considerable genetic similarity among varieties. These varieties /genotypes together made an internal group in all the dendrograms obtained in this study. This close similarity among these varieties /genotypes strongly supports the findings of Hirano (1977 and 1982) that the protein and isozyme profiles of *M. alba, M. latifolia,* and *M. bombycis* are so close that these species should be joined together under one species.

Furthermore, it is to be noted that the genetic variation at the DNA level is much more prominent than that at the protein level because of the codon degeneracy. Approximately 29% of mutations occurring at the nucleotide level cannot be detected by amino acid changes (Nei 1987). An additional 70-75% of amino acid substitutions cannot be detected by ordinary protein electrophoresis because of maintenance of net protein charge. In total, the detected genetic variation via allozyme is expected to be at least five to six fold less than at the DNA level (Nei 1987). Thus, the close relationships observe among these mulberry varieties is the true reflection of the genetic similarity present even at the DNA level. Thus, the taxonomists working on this aspect of mulberry should give serious thought in this direction by undertaking more detailed work to resolve the ambiguity over the separate species/varieties status of these genotypes. Similarly, the high fertility (>90%) obtained in controlled

hybridization among; Similar findings of Das and Krishnaswami (1965) on *M. Indica, M. alba, M. latifolia,* and *M. bombycis* supports the fact that these species should not be treated as separate species, as the very definition of species defined by Darwin (1859) emphasizes reproductive isolation of species. Furthermore, in most of the conventional methods of classifications, floral characteristic plays a major role in deciding the varieties identity of Texas. However, in mulberry, Mukherjee (1965) found a gradual reduction in one of the sexes on bisexual flowers leading to unisexuality. Likewise, Das and Mukherjee (1992) and Tikader et al. (1995) observed sex reversal upon hormonal application or pruning of branches of the plant. Minamizaw (1963) reported that high temperature, long day, and full daylight favored femaleness in mulberry.

These reports thus clearly suggest that floral characteristics cannot be taken as the sole diagnostic character for identification of species in mulberry. Therefore, it is clear that the classifications based on morphological, anatomical, or even biochemical characters alone do not identify the varieties accurately in a highly heterozygous plant like mulberry. Hence, it is essential to undertake detailed studies of this genus, using both biochemical, genetic, and morphofloral characteristics to resolve the confusion associated with species identity in mulberry.

The position of mulberry varieties in relation to other varieties needs special mention, as this method S-13, T-8, T-10 and T-12 , comprising was found to have an intermediate genetic relationship between the other group comprising RFS-135 and C-20 and T-12 When the total genotypes were analyzed individually, all varieties together into a separate cluster. However, when we analyzed the varietal variability, showed closer to the other group. Cross hybridization of different varieties of Morus showed produce*d* a high percentage (>80%) of fertile seeds, whereas a cross between some of mulberry varieties failed to develop some fertile seeds (Das and Krishnaswami 1965, Anonymous 1994). These findings, along with the result of the present investigation, suggest that as indicated in dendrogram 3,4,5,6,7, and 8. The similar observation made by Gururajan (1960), considering the morphological features of *M.alba* and *M. laevigata,* suggested that *M. indica* and *M. alba* are one species. Our findings on eighteen mulberry varieties with the molecular markers endorse this view to a certain extent, but considering the small number of varieties/ genotypes used for this analysis, it is desirable to undertake a detailed study with a greater number of genotypes to resolve the taxonomic position of this very important mulberry species.

The separate identity of mulberry varieties under different clusters as indicated in Figs. And S13 and T-8, T-10 and T-12 varieties are quite obvious from this study, as in all analyses, exhibited higher genetic distance from the other varieties. This is not surprising, since most of the genotypes reported under different ploids (Das 1961). However, in this study, we used diploid genotypes to avoid the differences pertaining to ploidy level. The floral characteristics of different mulberry varieties were also found to be different from others, as the length of the catkins in mulberry varieties varied from 4.1 to 7.2 cm, whereas that of the other varieties was in the range of 1.7 to 2.4 cm (Das *et al*. 1970, Tikader *et al*. 1995).

**Table.1** List of the primers used.

| Serial | Primer | Sequence(5´-3´) |
|---|---|---|
| 1 | OPA-0 1 | CAGGCCCTTC |
| 2 | OPA-03 | AGTCAGCCAC |
| 3 | OPA-13 | CAGCACCCAC |
| 4 | OPA-18 | AGGTGACCGT |
| 5 | OPB-17 | AGGGAACGAG |
| 6 | OPC-01 | TTCGAGCCAG |
| 7 | OPC-02 | GTGAGGCGTC |
| 8 | OPC-08 | TGGACCGGTG |
| 9 | OPC-10 | TGTCTGGGTG |
| 10 | OPC-12 | TGTCATCCCC |
| 11 | OPD-11 | AGCGCCATTG |
| 12 | OPD-13 | GGGGTGACGA |
| 13 | OPE-07 | AGATGCAGCC |
| 14 | OPE-19 | ACGGCGTATG |
| 15 | OPF-07 | CCGATATCCC |
| 16 | OPF-17 | AACCCGGGAA |
| 17 | UBC-807 | AGAGAGAGAGAGAGAGT |
| 18 | UBC-809 | AGAGAGAGAGAGAGAG |
| 19 | UBC-810 | GAGAGAGAGAGAGAGAT |
| 20 | UBC-811 | GAGAGAGAGAGAGAGA |

**Table.2** List of the RAPD primers used

| Serial No | Primer | Total number of bands | Total number of polymorphic bands |
|---|---|---|---|
| 1 | OPA-0 1 | 5 | 4 |
| 2 | OPA-03 | 4 | 4 |
| 3 | OPA-13 | 6 | 6 |
| 4 | OPA-18 | 5 | 4 |
| 5 | OPB-17 | 4 | 4 |
| 6 | OPC-01 | 5 | 0 |
| 7 | OPC-02 | 4 | 3 |
| 8 | OPC-08 | 4 | 3 |
| 9 | OPC-10 | 6 | 5 |
| 10 | OPC-12 | 3 | 2 |
| 11 | OPD-11 | 5 | 4 |
| 12 | OPD-13 | 6 | 6 |
| 13 | OPE-07 | 4 | 4 |
| 14 | OPE-19 | 4 | 3 |
| 15 | OPF-07 | 5 | 4 |
| 16 | OPF-17 | 5 | 5 |

**Table.3** List of the SSR primers used

| Serial No | Primer | Total number of bands | Total No of polymorphic bands |
|---|---|---|---|
| 1. | UBC-807 | 5 | 5 |
| 2. | UBC-809 | 6 | 6 |
| 3. | UBC-810 | 7 | 6 |
| 4. | UBC-811 | 6 | 6 |

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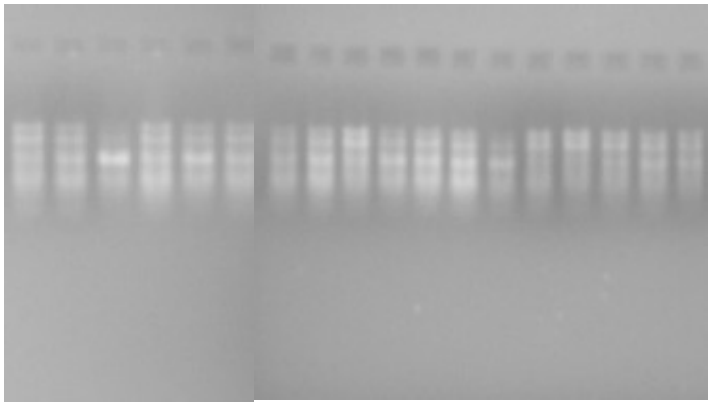
**Table.5** Summary of the RAPD AND SSR marker polymorphism

| **Marker** | **RAPD** | **SSR** |
|---|---|---|
| Total number of primers | 16 | 4 |
| Total number of bands amplified | 75 | 43 |
| Average number of bands per primer | 4.6 | 5.3 |
| Total number of polymorphic bands identified | 61 | 42 |
| Percentage of total bands which were polymorphic | 81.3 | 97.6 |
| Percentage of total bands which were polymorphic | 81.3 | 97.6 |

**Fig 1.**Gel profile of Mulberry genotypes amplified with primer OPE -07



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15161718

Fig 2. Dendrogram showing the relationship between the varieties es

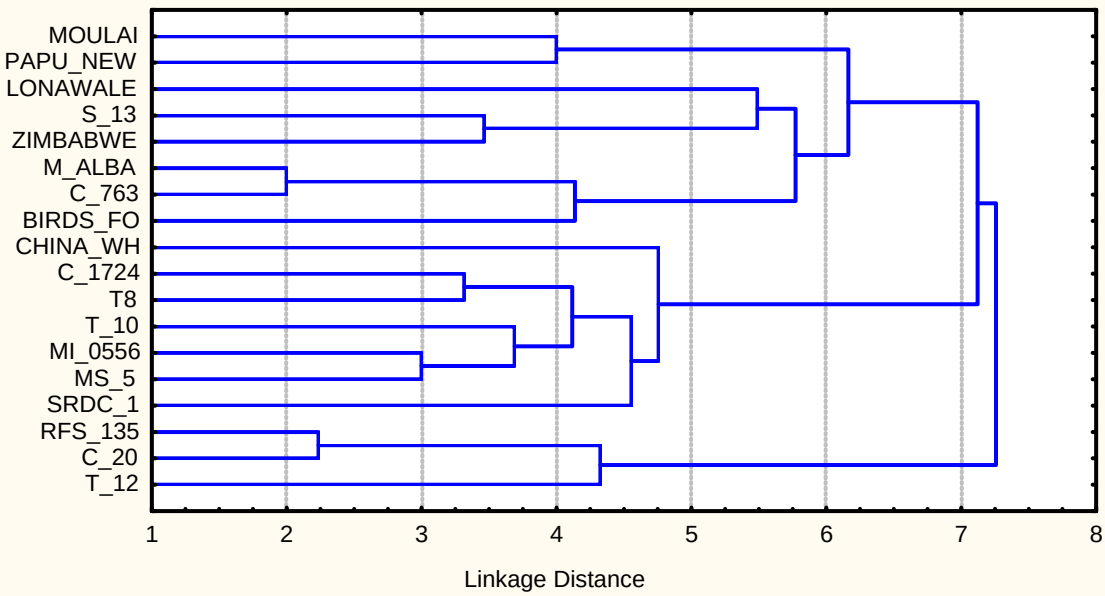
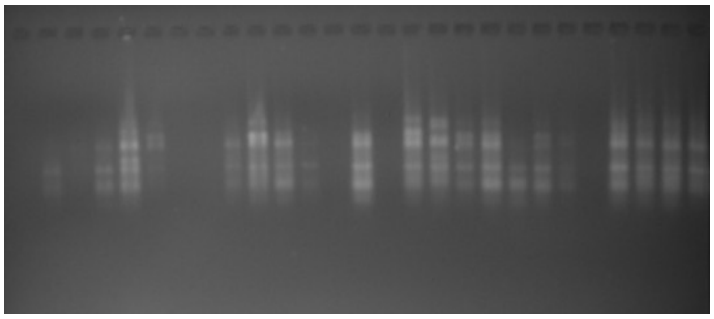



**Fig.3** Gel profile of Mulberry genotypes amplified with SSR primer UBC-809



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15161718

Similarly, controlled hybridizations between mulberry varieties failed to develop some fertile seeds (Anonymous 1994). These results, together with the findings of our study, clearly show that S-13 and T-8, T-10 and T-12 varieties *are* genetically different from other mulberry varieties and should continue to be considered a separate cluster.

The result presented in the present study demonstrated the utility of using RAPD and SSR markers to characterize genetic diversity among 18 promising varieties /cultivars /genotypes of mulberry. Differential polymorphism was noted in 18 cultivars of mulberry showing variation in percentage of polymorphic bands from 81.3% to 97.6% in using 16 primers RAPD and 4 SSR primers. The observed high proportion of polymorphic loci reveals profound intraspecific variation among the mulberry cultivars. Significant genetic variations by RAPD and SSR markers have also been reported in other species at the cultivar level

(Colombo *et al.* 1998, Das *et al.* 1998, Huang *et al.* 2003).

Wide genetic distances determined by Nei's, Dice and Jaccad genetic distance reveals relatively high genetic variation among 18 mulberry cultivars. The considerable polymorphism detected in the present study also illustrated the genetic diversity among mulberry cultivars of the same origin as reported among coffee cultivars (Sera *et al.* 2003). The observed intraspecific differences among 18 mulberry cultivars could be ascribed to the fluctuating micro and macro climatic conditions of habitat. Nei's Dice and Jaccad analysis of RAPD and SSR data also reveals that all mulberry cultivars belonging to the state of Karnataka are genetically closer and diversified other than the cultivars originally belonging to the distant habitat in the state of Karnataka. The greater sensitivity of RAPDs and SSR obtained from the results of mulberry cultivars, diversity may be derived from the rapid evolution of non-coding, repetitive DNA sequences detected by RAPD and SSR. This hypothesis has been corroborated from Plomion et al. (1995).

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