Evaluation Of Genetic Diversity In Mulberry Varieties Using Molecular Markers

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Abstract: Genetic relationships between twenty Mulberry varieties were assayed with Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers, which distinguish individuals, as well reflect the inherent variation and inter-relationships among the varieties. Sixteen decamer RAPD primers and 8 ISSR primers were used in the present study. Over 70 reproducible bands were generated by RAPD primers, out of which, 61 polymorphic bands were identified, conferring 81.3% polymorphism. Similarly 43 polymorphic bands were produced by ISSR primers, out of which, 42 polymorphic bands were identified conferring 97.6% polymorphism. All the primers produced typical banding in each of the cultivars, suggesting the applicability of this test in cultivar identification. Most of the individuals of the test exhibited to have unique molecular genotype. Population genetics structure analysis of these varieties further revealed high genetic differentiation coefficients (GST), the heterozygosity among population (Ht) showed with low gene flow (Nm) when 5th cluster was paired with other genotypes. On the basis of these parameters and the results of clusters analysis, it is concluded that three genotypes can be considered as a separate group of mulberry, whereas the other four clusters may be grouped separately. A dendrogram was constructed using Wards Euclidean distances and UPGMA method. Based on the number of bands, the genotypes were grouped to form 5 clusters.
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1.Introduction

Mulberry (M*orus* spp*)* belonging to the family Moraceae, is the sole source of food materials for silk worms *Bombyx mori* used in sericulture. The fruits of some species of mulberry being edible are used as an ingredient in the preparation of mulberry wine, jam and juice. The foliage, being nutritious and palatable, is used as cattle fodder. Some species of mulberry yield good quality wood in tropical climate. Mulberry is propagated asexually by stem cuttings or grafting. Some are propagated sexually using seeds. Although Mulberry is the native of China, white mulberry is cultivated throughout the world whereever silk worms are raised. Presently, *Morus L.* grows in warm climatic zones between 50° N latitude and 10° S latitude (Yokoyama 1962). So far there are about 150 species of genus *Morus* have been described (Vide index kewensis, London) and 50% of them have been considered as verities of the same species. Today Morus comprises about 68 recognized species and distributed in different countries mainly from Asian countries like Japan, China, India, Korea and Taiwan. Continental Asia and America are also rich in Morus species. Vavilov (1926), while reviewing the centers of origin placed genus *Morus L.* in China, Japan and Korean region. In India there are many species of *Morus alba, M. indica, M. serrata* and *M. laevigata* grown wild in the Himalayas. Several varieties have been introduced belonging to *M. multicaulis, M. nigra, M. sinensis* and *M. phillippinensis*. Most of the Indian varieties of mulberry belong to *M. indica*. The major area of mulberry cultivation is the tropical zone covering Karnataka, Andhra Pradesh and Tamil Nadu states. In the sub tropical zone, West Bengal, Himachal Pradesh and the North-Eastern states have major under mulberry cultivation (Ananda Rao 2002).

Mulberry bears different sex types, i.e., male, female and bisexual flowers on the same plant (Monoecious), or on different plants (dioecious) with expression of sex often depending on several physiological and biochemical factors (Das 1994). Through traditional breeding procedures, many improved varieties of mulberry trees have been developed today. There exist considerable differences of opinion in the classification of species of Morus by several systematics based on morphological characteristics. So there is a great need to identify the species at molecular level.

DNA finger printing techniques are quick and they accurately identify genetic difference among the cultivars. Since these techniques are not influenced by climatic or environmental factors, the results are genuine. The RAPD method relies on amplification of polymorphic DNA fragments by the polymerase chain reaction (PCR) using single oligonucleotide primer of arbitrary sequence. The RAPD method described by Williams et al. (1990) which is similar to the arbitrarily primed PCR (Welsh and McClellan 1990), is a fast and simple approach. The disadvantage of RAPD method is that markers are usually dominant. In addition, the reproducibility of RAPD banding patterns can be affected by different concentrations of reaction components and the cycle conditions (Williams *et a.l* 1992). RAPD technique has been shown to be useful in genetic analysis (Hu *et al.* 1991, Wilde *et al*. 1992), and phylogenetic analysis (Demeke *et al*. 1992, Halward *et al*. 1992, Kazan *et al.* 1993, Yu and Pauls 1993). Inter Simple Sequence Repeats (ISSR) is 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 to 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, Dongre *et al*. 2006, Basha and Sujatha 2007).Thus the present study, the first of it kind, was undertaken to investigate the interrelationships among twenty mulberry varieties extensively available in Karnataka. India.

2.Materials and Methods

Leaf samples of twenty mulberry varieties / accessions were collected from the germplasm maintained by the department of Sericulture, Bangalore University, G K V K and Thalaghatta pura, Bangalore. The first 2-4 leaves from the tips were collected and used for DNA extraction.

Isolation and purification of total genomic DNA was 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 supernatant collected 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 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) the PCR cycles and buffer compositions were as in chatterjee et al. (2004), sixteen primers such.OPA- 1, OPA-3, OPA-13, OPA-18, OPB-17, OPC-01, OPC-02, OPC-08, OPC-10, OPC-12, OPD-11, OPD-13, OPE-07, OPE-19, OPF-07, and OPF-17. Amplification reactions were carried out in 25 µl reaction mixture containing template DNA (30 ng),10pmol of primer (Operon technologies USA, Inc.), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl,1U *Taq* DNA polymerase (Hi-media) and 200 µM of each dNTPs (Hi-media). Amplification was performed in the Corbett Research PCR machine (CG1-96) for 35 cycles after an initial denaturation at 94ºC for 7 min. In each cycle, denaturation for 1 min at 94 ºC, annealing for 1 min at 35 ºC and extension for 2 min at 72 ºC was programmed with a final extension step at 72 ºC for 5 min. Amplified DNA fragments were separated out on 1.4% agarose gel stained with ethidium bromide. Running buffer containing Tris-buffer, Boricacid and EDTA (pH 8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 25 µl reaction volume and 5µl of loading buffer (Sucrose, Bromophenol blue and Xylenecyanol) together. Electrophoresis was conducted at 70 volts for 3 hours and the gel photographed under UV light using gel dock system (Herolab).

ISSR:

PCR amplification was carried out according to Zietkiewicz et al. (1994). ISSR analysis of genomic DNA of 45 varieties/ accessions was carried out using 8 selected ISSR primers (UBC- 807, 809, 810, 811, 812, 820, 825, 828), obtained from 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 were : 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 16 primers with 100 to 3000 base pairs marker level pair wise. Squared Euclidean distance and UPGMA were used to calculate the distances, varieties were clustered following Ward's method of Statistics version 5.0 a computer application and UPGMA method were used to generate dendrogram.

Genetic relationship among genotypes

In the first part of the study, the genetic relationships and their grouping on the basis of the RAPD and ISSR markers were assessed using Nei's coefficients *(S* = 2ab/(Na + *Nb))* (Nei and Li 1979) and Dice's coefficients *(S = 2Nab/(2Nab + Na + Nb))* (Sneath and Sokal 1973), where *Nab* is the number of bands that are shared by genotypes *a* and *b,* Na is the number of bands present in *a,* and *Nb* is the number of bands present in *b;* as well as Jaccard's coefficients *(S* = NAB(NAB + *N*a + *Nb))* (Jaccard 1901), where *Nab* is the number of bands that are shared by genotypes *a* and *b, Na* is the number of bands present in *a* and absent in *b,* and *NB* is the number of bands present in *b* and absent in *a.*

Dissimilarity matrices were calculated from all of the above similarity matrices and dendrograms were generated using Wards Euclidean methods and UPGMA,(Sneath and Sokal 1973) on PHYLIP 3.5c software program (Felsenstein 1993). The robustness of the dendorgram was tested by estimating cophenetic correlation for each dendrogram and comparing it with the original genetic dissimilarity matrix, using Mantel's matrix correspondence test (Mantel 1967). Computing the cophenetic values and constructing the cophenetic matrices for each set of data, the differences between the dendrograms based on RAPD and ISSR and their pooled markers were assessed. These matrices generated from different methods were also tested for correlations using Mantel's test for matrix correspondence (Mantel 1967).

Further, relationships among the varieties were also investigated by analyzing the genotypes of each variety as a single population. The inter and intragenetic divergence was estimated using RAPD and ISSR markers. All the coefficients were also worked out for each variety and dendrogram were constructed to find out the genetic relationships among the varieties. The variability in the population was also calculated using Nie s (Nei 1973) coefficient of gene differentiation (Gst) in POPGENE, version 1.3 (Yeh 1998). In POPGENE, the genetic divergence among different populations is calculated using a multiallelic analogue of Fst among a finite number of populations, which is otherwise called the coerricient of gene differentiations, (Nei1973). This is stated in the following equation.

$$G_{ST} = D_{ST}/H_t = (H_t - H_s)/H_t$$

Where Dst is the average gene diversity between sub-populations, including the comparisons of subpopulations with themselves, with $D_{ST} = (H_t - H_s)$. G_{ST} is an extension of Neis (1972) genetic distance between a pair of populations to the case of hierarchical structure of populations (Nei 1973). Ht is defined by the following equation:

$$H_t = 1 - \Sigma Pi^2$$

Where Pi is the frequency of the allele at a locus in a population. Hence, H_s was defined in terms of gene diversities. However, for random mating subpopulations, gene diversities can be defined as expected heterozygosities under Hardy-Weinberg averaged among population (H_s) and of the total population (H_t). The main difference between G_{ST} and F_{ST} (Wright 1943) is that in G_{ST} the estimation of the heterozgosities relies on allele frequencies (Nei 1987), whereas in F_{ST}, to estimate the H_s, the individual genotypes have to be known. The estimate of gene flow from G_{ST} was calculated as

$$N_m = 0.5(1- G_{ST}) / G_{ST}$$

3. Results

The procedure described earlier gave a good yield 1000-3000 ng per µl for every 2 g of leaf sample. The DNA obtained was of high quality and responded well for the amplification reactions. Spectrophotometer reading of 1.4-1.8 (260nm/280nm) confirmed the quality of DNA. The quality of the DNA was also confirmed by gel electrophoresis.

The amplification conditions were optimized in a pilot experiment to get high quality, intense, repeatable banding patterns, with varying concentrations of Mg^{2+} ions, primer and template DNA. An increase in the concentration of Mg2 primers gave clear, intense bands. But the increase in DNA concentration had no effect at least till 50ng/25µl.

In this study, a total of 120 arbitrary decamer, single stranded primers (OPA to OPF with 20 primers in each group) were utilized to screen the genomic DNA of mulberry genotypes. Among them, 28 primers on an average gave six bands. 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 show a representative DNA fingerprint generated by the RAPD and ISSR primers-809, UBC-811, UBC-830, UBC-835, and UBC-86 and table-2 and 3 and 4 gives the list of random primers used for the present study. A dendrogram based on Ward's and UPGMA method of analysis (Fig. 3, 4, 5, 6, 7 and 8).

DNA polymorphism within the genotype

The analysis of the DNA extracted from twenty mulberry genotypes, from each variety with 8 selected ISSR primers (UBC- 807, 809, 810, 811, 812, 820, 825, 828), and Sixteen primers RAPD such.OPA- 1, OPA-3, OPA-13, OPA-18, OPB-17, OPC-01, OPC-02, OPC-08, OPC-10, OPC-12, OPD-11, OPD-13, OPE-07, OPE-19, OPF-07, and OPF-17 showed DNA polymorphism among the varieties of the genotype. Hence, for further studies, DNA from a single plant was used for each genotype.

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, out 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 2 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 pattern. Further, the isolation and gel profiling system shows variation in amplification because of the primer sequence. The average number of bands number of bands per primers was 4.6 and percentage of polymorphic was 81.3%

Genetic similarity among the 20 mulberry varieties were estimated on the basis of the ISSR banding profiles generated by each primer with the genomic DNA of the mulberry varieties. The genomic DNA of these mulberry varieties which were amplified with oligonucleotide primer UBC - 809 generated a total number of 108 ISSR bands. The number of ISSR is specific to each mulberry variety showing a 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, 19 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, 20 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 a very high ISSR banding pattern to distinguish in mulberry varieties as a diverse character the identification of ISSR banding pattern is important because it follows Mendelian inheritance character, despite, some the varieties showing thin ISSR banding patterns on gel profile. Despite some of the genomic DNA of mulberry varieties revealed no ISSR banding pattern in varieties such as 2,6,7,12,14, the data clearly indicates diverse distribution of ISSR 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 8 ISSR primers UBC-809 clearly revealed such variability among the genotypes **(**Fig. 2**).** The 8 ISSR primers generated a total of 43 bands, of which 42 were polymorphic, generating 97.6 % polymorphism among the 20 mulberry genotypes.

Genetic similarity among genotypes

The genetic similarity coefficients among genotypes estimated on the basis of Nei and Li (1979) varied from 0.987 to 0.533 with an average genetic similarity of 0.760 in RAPD markers. The same was in the range of 0.923 and 0.532 with an average of 0.727in ISSR. The Dice coefficients (Sneath and Sokal 1973) among the genotypes also showed considerable variation. In RAPD, it varied from 0.460 to 0.373 and in ISSR it was between 0.475 and 0.362. The similarity coefficient among the genotypes estimated on the basis of Jac (1901) was between 0.842 and 0.333, with an average Of 0.587

in RAPD and 0.922, 0.443 and 0.682 in, ISSR, and in the pooled data RAPD + ISSR markers, revealed 0.923, 0.632 with an average of 0.727, and 0.472, 0.374 with an average of 0.423, and 0.832, 0.232 with an average of 0.532 respectively. The Pearson's cortion coefficients between different matrices subjected to Mantel test (Mantel 1967) were found to be highly significant *(r* = 0.435-0.998, *p* = 0.000) (Table 5).

The dendrograms realized from the above matrices, RAPD ISSR, and the pooled data from both markers using Wards methods of Euclidean distance and UPGMA method grouped the 20 mulberry genotypes into three, four and five clusters (Fig. 3, 4,5,6,7 and 8). The dendrogram of the both RAPD and ISSR data revealed genetic diversity and relationships of twenty mulberry varieties using UPGMA method. The dendrogram of RAPD of twenty mulberry showed three clusters. The cluster one included varieties such as S13, S146, S34, V1, DD and *M rotund* which are not related with each other, where as varieties *M lhonse* showed relationship with Karanahalli local . The variety Srinagar showed relationship with S36, where as other varieties are diversified with one another (Fig.3). The varieties Karanahalli local showed relationship with *M.lhonse*, where as other varieties revealed diversity between each other (Fig. 4).

The dendrogram of ISSR data 20 mulberry varieties revealed that Karanahalli local, S1635, Mysore local, V1, and S146 are related with each other (Fig.5). In dendrogram Karanahalli local, Srinagar, S1, Mysore local, V1, S145 and S36 Fig-6. The RAPD and ISSR data revealed that .*M.macrou,* Srinagar, S1, *M.lhonse*, MR2, s1635, S36, R127, Newvar20, Karanahall Mysore local, V1 and S146 are inter related with other(Fig.7). Further, Fig. 8 revealed that all the varieties interrelated with other except a few varieties.

Another important point noticed from the dendrogram is the high genetic distance enjoyed in some mulberry varieties from the others while the genotypes of other varieties showed closer relationships. The Mai tests (Mantel 1997) between cophenetic correlation mal and its corresponding similarity matrix were found very significant *(r* = 0.535-0.842, *p* = 0.000) for all dendrogram (Table 6).

The dendrogram of the both RAPD and ISSR data revealed genetic diversity and relationships of twenty mulberry varieties using UPGMA method. The dendrogram of RAPD of twenty mulberry showed three clusters. Cluster one included varieties such as S13, S146, S34, V1, DD and *M. rotund* which are not related with each other, where as varieties *M. lhonse* showed relationship with Karanahalli. The variety Srinagar showed relationship with S36, where as other varieties are diversified with one another (Fig. 3).The dendrogram based on RAPD data revealed that S1, S36, Chinapeaking, *M. macrou*, Srinagar, Karanahalli and *M. lhonse* are related with each other (Fig. 4). Similar Observation was made on ISSR data (Fig. 5, 6, 7, and 8).

4. Discussion

The results of the present investigation clearly demonstrate the usefulness of RAPD and ISSR to delineate the interrelationships among varieties/ genotypes of 20 mulberry varieties present in Karnataka, India. Although ISSR primers unraveled more polymorphism than RAPD primers, both RAPD and ISSR primers generated almost similar types of genetic relationships among the genotypes and their respective varieties. Using ISSR 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 ISSR 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 the three types of matrix and their corresponding dendrograms showed more or less similar results. However, from the dendrograms it is clear that 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 mulberry for phylogenetic studies.

Regarding the genetic relationships of varieties / genotypes, the pair-wise 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 *M. laevigata* as a separate cluster indicated its greater genetic divergence from other species. 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 protein electrophoresis because of the 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 observed 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 a more detailed work to resolve the ambiguity over the separate species status of these genotypes. Similarly, the high fertility (>90%) obtained in controlled hybridization among them.

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 play a major role in deciding the varieties identity of taxa. 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 biochemical, genetic, and morphofloral characteristics to get over the confusion associated with species identity in mulberry.

The position of mulberry varieties in relation to other varieties needs special mention, as this method *M. lhonse,* and Karanahalli comprising was found to have an intermediate genetic relationship between the other group comprising S1, R-127, S1635, and Srinagar, *M. macru* and S36 When the total genotypes were analyzed individually, all varieties together into a separate cluster. However, when the varietal variability was analyzed showed more closeness 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 any 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 Earlier, similar observation made by Gururajan (1960), considering the morphological features of *M. indica, M. alba,* and *M. laevigata,* suggested that *M. indica* and *M. alba* are one species. Our findings on twenty 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 identify the taxonomic position of this very important mulberry species.

The separate identity of mulberry varieties under different clusters as indicated in Figs. and *S1* and S36 varieties is 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 that 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 4.2 to 7.3 cm, whereas that of the other varieties was in the range of 1.8 to 2.5 cm (Das *et al*. 1970, Tikader *et al*. 1995). Similarly, controlled hybridizations between mulberry varieties failed to develop any fertile seeds (Anonymous 1994). These results, together with the findings of our study, clearly show that S and V 1 varieties *is* genetically different from other mulberry varieties and should continue to be considered as separate clusters.

Table 1. The list of 20 mulberry varieties / accessions used for the present study

Serial No	SMGS Acc-No	Name	Donor Name	Donor Id.No.	Country/ State	Origin
1.	MI-0012	S-13	RSRS, Kodathi	--	KAR	OPH selection
2.	MI-0045	S-146	RSRS, Kodathi	208	KAR	OPH selection
3.	MI-0160	S-34	CSR&TI, Mysore	--	KAR	Mutation
4.	MI-0308	V-1	RSRS, Kodathi	--	KAR	CPH
5.		M-5				
6.	MI-0052	Mysore local	CSR&TI, Mysore	144	KAR	Selection
7.	MI-0066	R-175				
8.	MI-0021	DD	KSSRDI,Bangalore	--	UPR	Collection
9.	MI-0423	Srinagar	Expl. North West India	AT96(1)29	UPR	Collection
10.	MI-0025	MR-2	RSRS, Kodathi	111	TNU	Clonal selection
11.	MI-0173	S-1635	CSR&TI,Berhamp-ore	--	WBL	OPH selection
12.		Karanahalli local				
13.	MI-0013	S-36	CSR&TI, Mysore	157	KAR	Mutation
14.	ME-0050	*M. macroura*	CSR&TI, Mysore	307	JPN	Collection
15.	ME-0095	*M. rotundiloba*	CSR&TI, Mysore	295	BUR	Collection
16.	ME-0005	Chinapeaking	RSRS, Kodathi	147	PHI	Clonal selection
17.	ME-0107	*M. lhou-seringe*	CSR&TI,Berhampore	--	FRA	Collection
18.	ME-0065	S-1	CSR&TI, Mysore	139	BUR	Clonal selection
19.	MI-0024	Assambola	CSR&TI, Mysore	105	ASM	Clonal selection
20.	MI-0047	S-41	CSR&TI, Mysore	158	KAR	Mutation

Table 2. 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	AGAGAGAGAGAGAGAGG
19	UBC-810	GAGAGAGAGAGAGAGAT
20	UBC-811	GAGAGAGAGAGAGAGAA
21	UBC-812	GAGAGAGAGAGAGAGAA
22	UBC-814	CTCTCTCTCTCTCTCTA
23	UBC-825	ACACACACACACACACT
24	UBC-830	TGTGTGTGTGTGTGTGG

Table 3. List of the RAPD primers used.

Serial No	Primer	Total number of	No. of polymorphic
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 4. The genetic similarity coefficient estimated from the RAPD and ISSR and pooled markers in 20 genotypes of Mulberry.

	Maximum	Minimum	Mean
RAPD			
Nei and Li	0.987	0.533	0.760
			0.416
Dice	0.460	0.373	0.587
Jaccard	0.842	0.333	
ISSR			0.727
Nei and Li	0.923	0.532	
Dice	0.475	0.362	0.420
Jaccard	0.922	0.443	
			0.682
RAPD+ISSR	0.922	0.632	0.727
Nei and Li			
Dice	0.472	0.374	0.423
Jaccard	0.832	0.232	0.532

Table 5. The Nei's genetic heterozygosity estimated among 20 mulberry varieties

M ulberry varieties	**No. of observed alleles**	**No. of effective alleles**	**Genetic Heterozygosity**	**Shannon's Information Index**
RAPD				
S-13	1.38 ± 0.48	1.26 ± 0.38	0.14 ± 0.19	0.23 ± 0.28
S-146	1.42 ± 0.50	1.27 ± 0.37	0.15 ± 0.20	0.23 ± 0.88
S-34	1.42 ± 0.50	1.23 ± 0.34	0.13 ± 0.18	0.21 ± 0.27
V-1	1.42 ± 0.50	1.30 ± 0.38	0.16 ± 0.20	0.24 ± 0.29
M-5	1.45 ± 0.50	1.28 ± 0.38	0.14 ± 0.20	0.28 ± 0.29
Mysore local	1.36 ± 0.42	1.19 ± 0.34	0.12 ± 0.18	0.17 ± 0.26
R-175	1.25 ± 0.42	1.14 ± 0.30	0.08 ± 0.17	0.14 ± 0.25
DD	1.34 ± 0.43	1.22 ± 0.35	0.14 ± 0.18	0.18 ± 0.27
Srinagar	1.30 ± 0.46	1.18 ± 0.32	0.12 ± 0.18	0.16 ± 0.26
MR-2	1.45 ± 0.54	1.27 ± 0.36	0.15 ± 0.19	0.266± 0.28
S-1635	1.45 ± 0.47	1.27 ± 0.36	0.16 ± 0.19	0.23 ± 0.28
Karanahallilocal	1.38 ± 0.47	1.24 ± 0.34	0.14 ± 0.19	0.23 ± 0.27
S-36	1.44 ± 0.49	1.28 ± 0.36	0.18± 0.20	0.24 ± 0.27
M. macroura	1.46 ± 0.49	1.26 ± 0.33	0.15 ± 0.19	0.23 ± 0.28
M. rotundiloba	1.67 ± 0.46	1.45 ± 0.36	0.25 ± 0.19	0.35 ± 0.27
Chinapeaking	1.48 ± 0.49	1.26 ± 0.36	0.15 ± 0.18	0.24 ± 0.24
M. lhou- seringe	1.36 ± 0.48	1.23 ± 0.33	0.13 ± 0.19	0.22 ± 0.25
S-1	1.48 ± 0.49	1.28 ± 0.36	0.176± 0.20	0.25 ± 0.26
Assambola	1.46 ± 0.49	1.26 ± 0.34	0.16 ± 0.19	0.24 ± 0.24
S-41	1.67 ± 0.46	1.40 ± 0.36	0.24 ± 0.19	0.35 ± 0.25

Table 6. The Nei's genetic heterozygosity estimated among 20 mulberry varieties

M mulberry varieties	No. of observed alleles	No. of effective alleles	Genetic Heterozygosity	Shannon's Information Index
ISSR				
S-13	1.49 ± 0.49	1.24 ± 0.35	0.15 ± 0.19	0.22 ± 0.28
S-146	1.42 ± 0.50	1.24 ± 0.37	0.16 ± 0.20	0.23 ± 0.88
S-34	1.40 ± 0.50	1.24 ± 0.34	0.14 ± 0.18	0.21 ± 0.27
V-1	1.44 ± 0.50	1.34 ± 0.38	0.17 ± 0.20	0.25 ± 0.29
M-5	1.41 ± 0.50	1.24 ± 0.37	0.15 ± 0.20	0.23 ± 0.29
Mysore local	1.43± 0.42	1.14 ± 0.33	0.11 ± 0.18	0.16 ± 0.26
R-175	1.24± 0.42	1.14 ± 0.31	0.09 ± 0.17	0.13 ± 0.25
DD	1.44 ± 0.47	1.24 ± 0.35	0.13 ± 0.18	0.19 ± 0.27
Srinagar	1.40 ± 0.46	1.14 ± 0.33	0.11 ± 0.18	0.17 ± 0.26
MR-2	1.46 ± 0.50	1.24 ± 0.37	0.16 ± 0.19	0.24 ± 0.28
S-1635	1.45 ± 0.49	1.24 ± 0.36	0.16 ± 0.19	0.24 ± 0.28
Karanahalli local	1.48 ± 0.48	1.26 ± 0.34	0.14 ± 0.19	0.22 ± 0.27
S-36	1.45 ± 0.49	1.28 ± 0.37	0.17 ± 0.20	0.25 ± 0.27
M. macroura	1.44 ± 0.49	1.27 ± 0.35	0.16 ± 0.19	0.24 ± 0.28
M. rotundiloba	1.67 ± 0.46	1.43 ± 0.37	0.24 ± 0.19	0.36 ± 0.27
Chinapeaking	1.45 ± 0.49	1.26 ± 0.36	0.16 ± 0.19	0.24 ± 0.28
M. lhou- seringe	1.48 ± 0.48	1.26 ± 0.34	0.14 ± 0.19	0.22 ± 0.27
S-1	1.46 ± 0.49	1.26 ± 0.37	0.17 ± 0.20	0.25 ± 0.27
Assambola	1.45 ± 0.49	1.26 ± 0.35	0.16 ± 0.19	0.24 ± 0.28
S-41	1.48 ± 0.46	1.26 ± 0.37	0.24 ± 0.19	0.36 ± 0.27

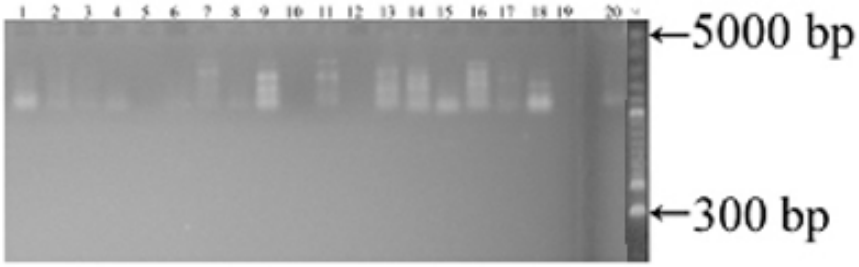

Fig.1: Gel profile of 20 varieties of mulberry amplified with RAPD primers OPD - 11

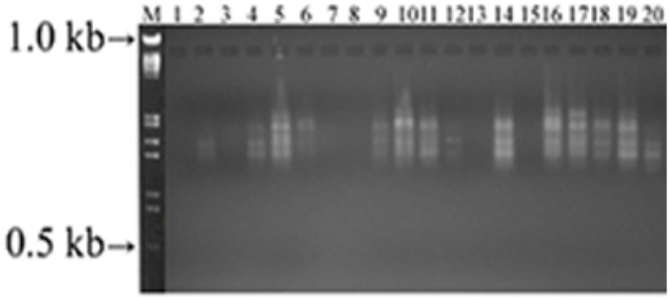

Fig.2: Gel profile of 20 varieties of mulberry amplified with ISSR primer UBC - 809

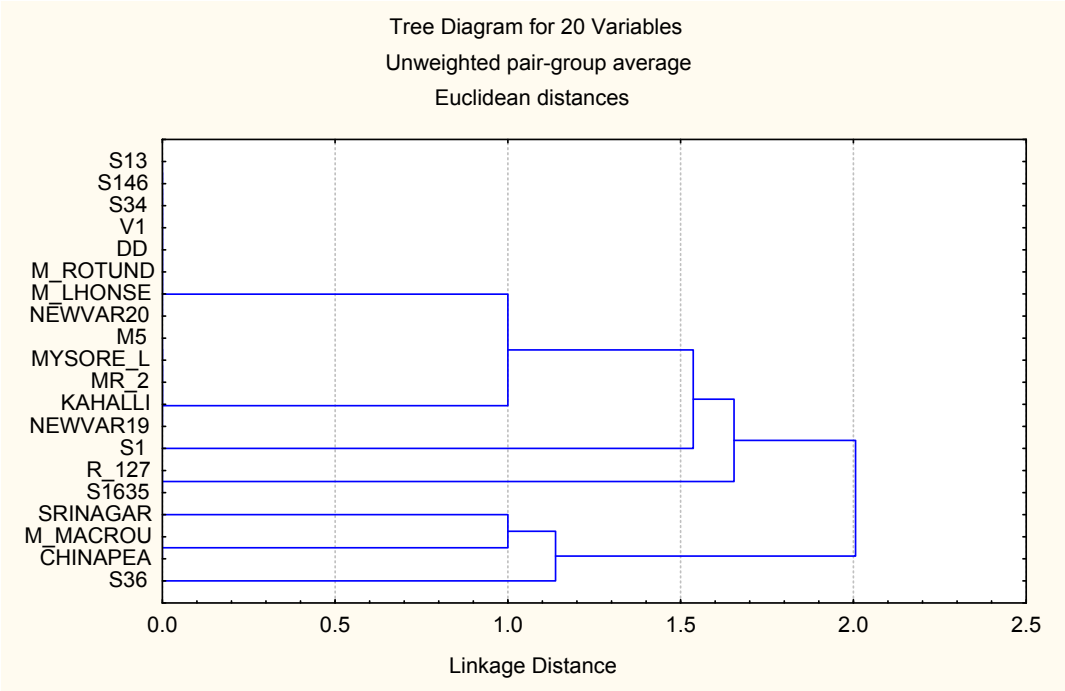

Fig 3. Cluster analysis of 20 mulberry varieties generated by UPGMA method for cumulative band data obtained by RAPD data

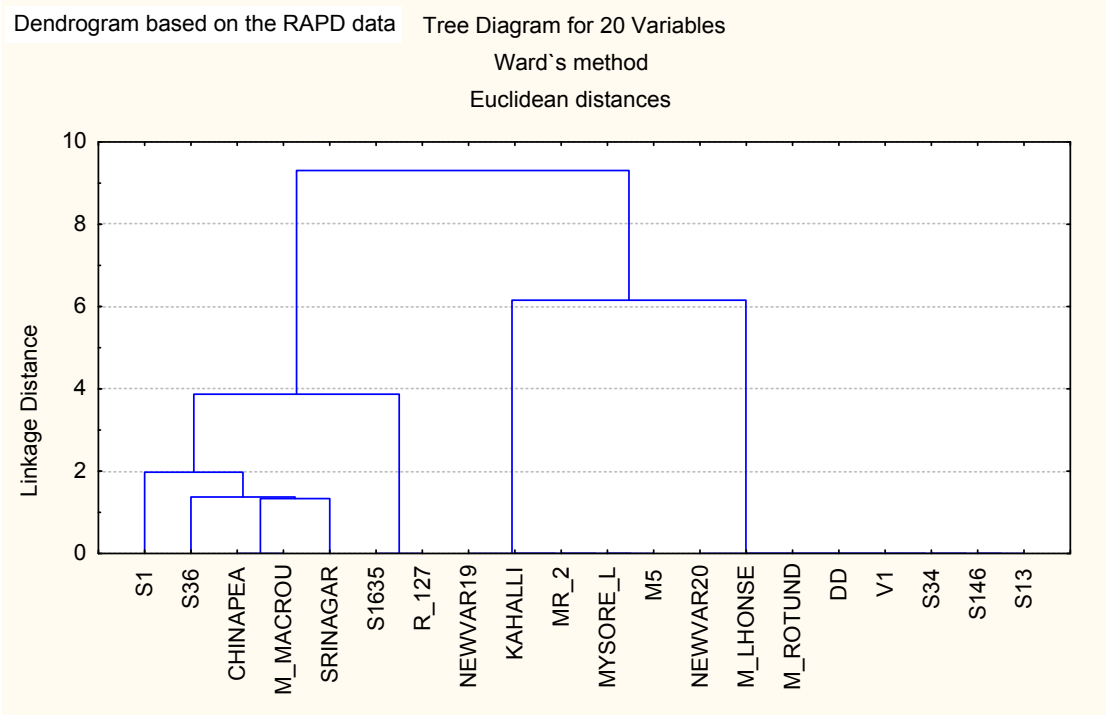

Fig 4. Dendrogram based on based on RAPD data of 20 mulberry varieties generated by Wards method

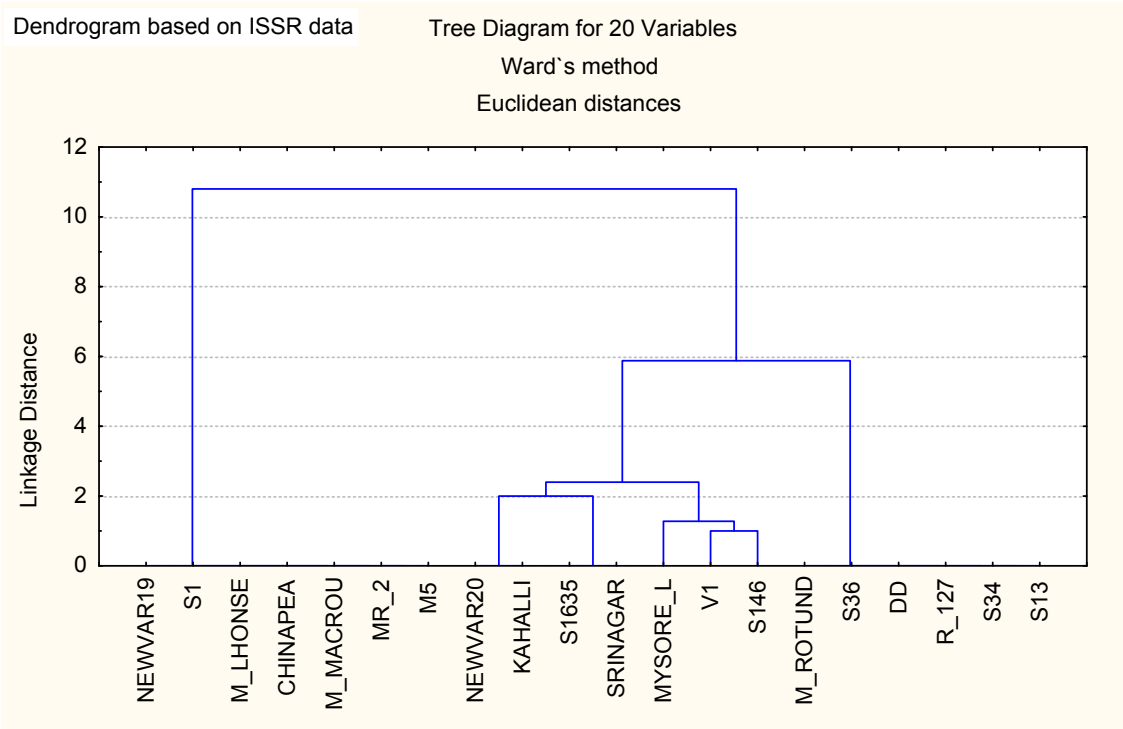

Fig 5. Dendrogram based on based on ISSR data of 20 mulberry varieties generated by Wards method

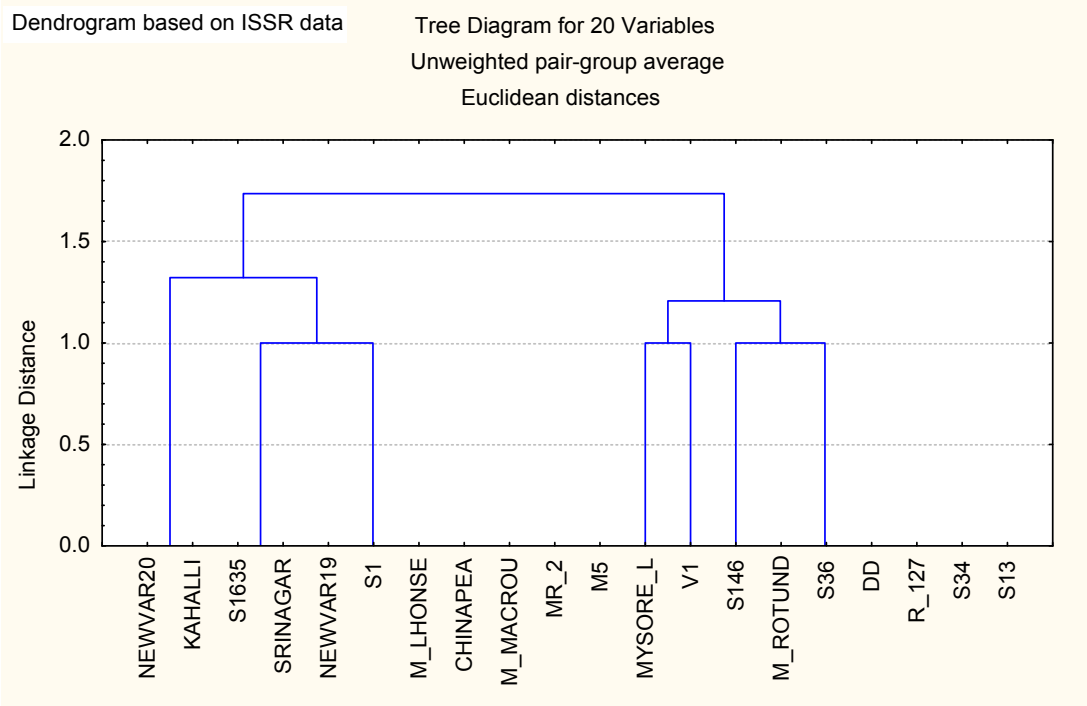

Fig 6. Dendrogram based on based on ISSR data of 20 mulberry varieties generated by UPGMA method

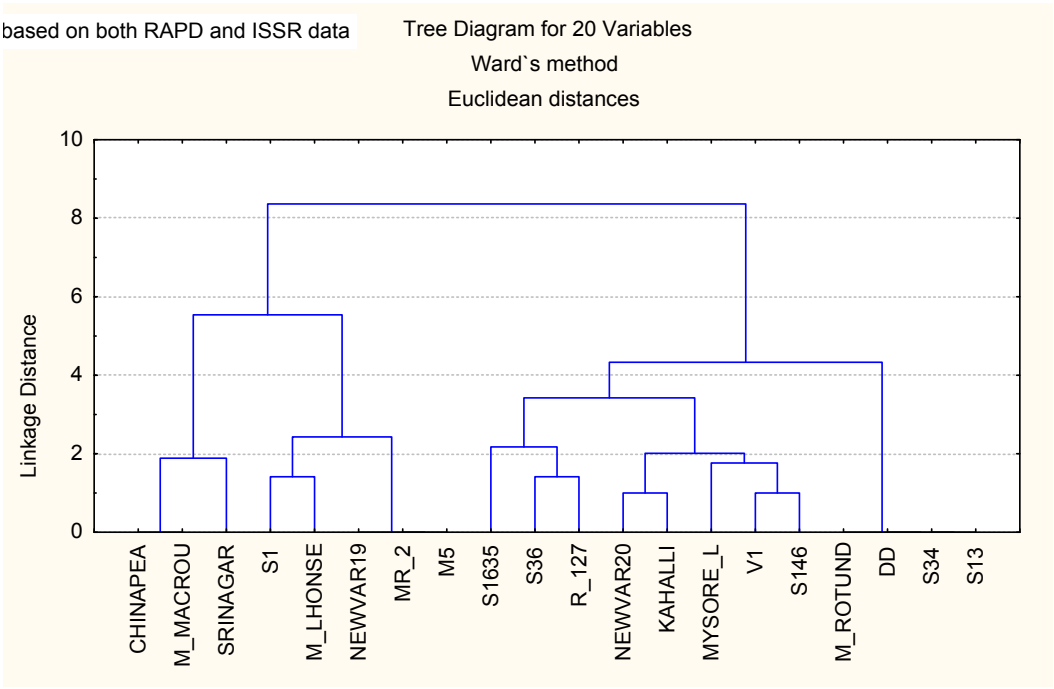

Fig 7. Dendrogram based on based on RAPD and ISSR data of 20 mulberry varieties generated by Wards method

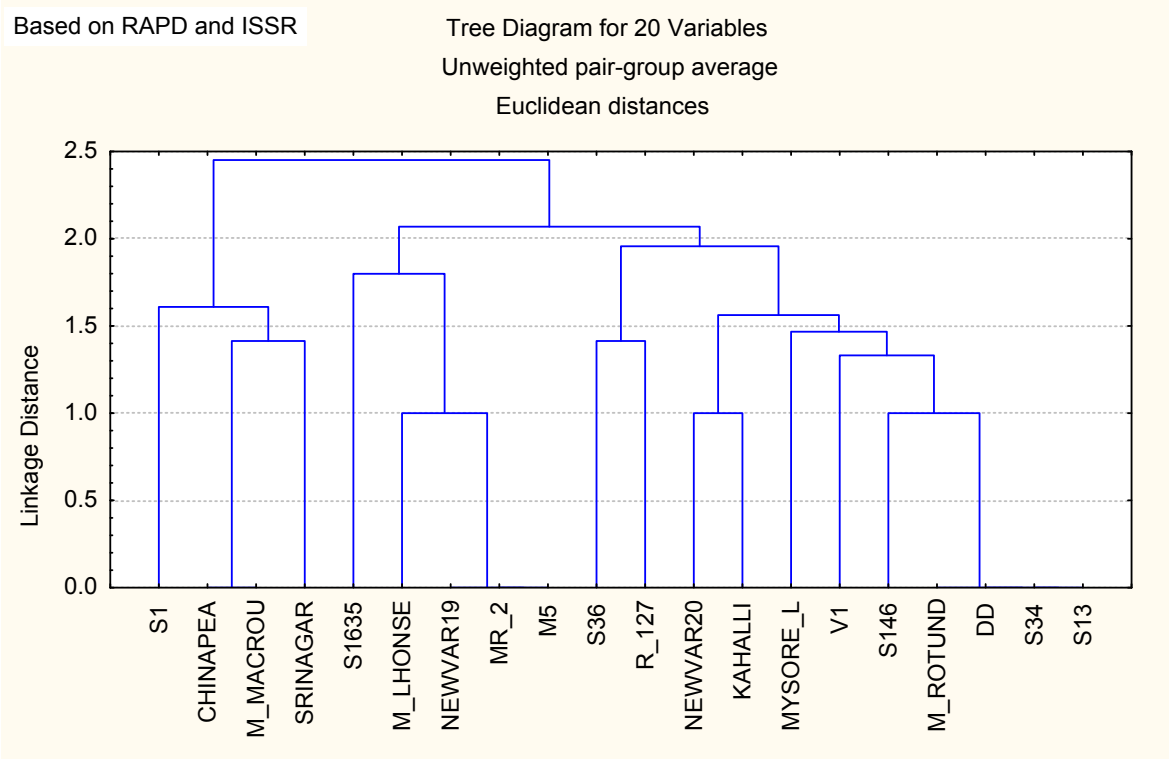

Fig 8.Dendrogram based on based on RAPD and ISSR data of 20 mulberry varieties generated by UPGMA method

The population structure analyses further demonstrated the genetic difference of twenty mulberry varieties and the closer relationships the total heterozygosity within the population (Ht) and between populations *(Dst)* and the genetic differentiation coefficients (Gst) were much higher than the same between other varieties. These values clearly suggest higher genetic divergence of *S* varieties from the other varieties. This is further evidenced from the low gene flow (Nm) from some of the mulberry varieties *to* the other.

The exchange of genes between populations of homogenizes allele and frequencies between populations determine the relative effect of selection and genetic drift. High gene flow between populations precludes local adaptation and also impedes the process of speciation (Barton and Hewitt 1985). In population genetics, a value of gene flow (Nm) < 1.0 (fewer than one migrant per generation into a population) or equivalently, a value of gene differentiation (G_{ST}) > 0.25 is generally regarded as the threshold quantities beyond which significant population differentiation occurs (Slatkin 1987) (Table 5,6,7,8,9, and 10).

The very low Nm present in S varieties further reflects the reproductive isolation it holds from other varieties / genotypes of mulberry. Similar observation was made by Darwin 1859 since reproductive isolation is one of the important criteria considered for species recognition (Darwin 1859) this can be treated as a separate species under the genus *Morus*. Thus, from the overall studies, it can be concluded that the mulberry varieties / genotypes boundary is not very rigid in mulberry; identification of taxa based on morphofloral characters alone often generates misleading results. Thus, an approach integrating morphological, biochemical, and genetic and (or) molecular parameters is required to resolve the problems pertaining to the taxonomic positions of most of the presently designated varieties / genotypes in mulberry. Furthermore, S and other mulberry varieties can be considered to be a separate group of

mulberry, while the other varieties may be joined together and treated as separate group etc.

The result presented in the present study demonstrated the appropriacy of using RAPD and ISSR markers to characterize genetic diversity among 20 promising varieties /cultivars /genotypes of mulberry. Differential polymorphism was noted in 20 cultivars of mulberry showing variation in percentage of polymorphic bands from 81.3% to 97.6% in using 16 primers RAPD and 8 ISSR primers. The observed high proportion of polymorphic loci reveals profound intraspecific variation among the mulberry cultivars. Significant genetic variations by RAPD and ISSR markers have also been reported in other species at 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 20 mulberry cultivars. The considerable polymorphism detected in the present study also illustrated genetic diversity among mulberry cultivars of the same origin as reported among coffee cultivars (Sera *et al.* 2003). The observed intraspecific differences among 20 mulberry cultivars could be ascribed to the fluctuating micro and macro climatic conditions of habitat. Nei's Dice and Jaccad analysis of RAPD and ISSR 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 ISSR obtained in the results of mulberry cultivars, diversity may be derived from rapid evolution of non-coding, repetitive DNA sequences detected by RAPD and ISSR. This hypothesis has been corroborated by Plomion et al. (1995).

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