Single primer amplification reaction methods reveal exotic and indigenous mulberry varieties are similarly diverse[§]

ESHA BHATTACHARYA[†,1], S B DANDIN[‡,2] and SHIRISH ANAND RANADE[†,*]

[†]*Plant Molecular Biology (Genomics), National Botanical Research Institute, Lucknow 226 001, India*
[‡]*Central Sericultural Germplasm Resources Center, Central Silk Board, Hosur 635 109, India*
Present Addresses: [1]*Department of Genetics, University of Delhi South Campus, New Delhi 110 021, India*
[2]*Central Sericulture Research and Training Institute, Mysore 570 008, India*

**Corresponding author (Fax, 91-522-2205836; Email, shirishranade@yahoo.com)*

Mulberry is the sole food source for mulberry silkworm and a number of indigenous and exotic varieties are used in sericulture. Studies on assessment of genetic diversity have been done amongst a few mulberry varieties using one or at the most two methods. However, no comprehensive study on a large number of varieties has been carried out. In present study, single primer amplification reaction (SPAR) methods have been used for determination of diversity in 27 mulberry varieties (exotic as well as indigenous), using four minisatellite core sequence primers for directed amplification of minisatellite DNA (DAMD), three simple sequence repeat (SSR) motifs as primers for inter simple sequence repeat (ISSR) and 20 arbitrary sequence decamer primers for random amplified polymorphic DNA (RAPD) reactions. The Jaccard coefficients were determined for the DAMD, ISSR and RAPD band data (total of 58, 39 and 235 bands respectively). All three methods revealed wide range of distances supporting a wide range of mulberry genetic diversity. A cumulative analysis of the data generated by three methods resulted in a neighbour-joining (NJ) tree that gave a better reflection of the relatedness and affinities of the varieties to each other. Comparison of the three methods by marker indices and the Mantel test of correlation indicated that though all methods were useful for the assessment of diversity in mulberry, the DAMD method was better. When considered as two groups (10 exotic and 17 indigenous varieties), the mulberry varieties in the exotic group were found to have slightly greater diversity than the indigenous ones. These results support the concept of naturalization of mulberry varieties at locales distant from their origins.

[Bhattacharya E, Dandin S B and Ranade S A 2005 Single primer amplification reaction methods reveal exotic and indigenous mulberry varieties are similarly diverse; *J. Biosci.* **30** 669–677]

1. Introduction

The genus *Morus* L., commonly known as mulberry, belongs to the family Moraceae of the order Urticales. It is better known as the exclusive food source of *Bombyx mori* L., the mulberry silkworm, though other species are also used for fodder, as a source of edible fruits and as herbal remedies. Mulberry is an ancient plant and has presumably originated in China or India. Thereafter, the global silk trade may have resulted in mulberry being widely distributed. Despite economic importance, the origin and domestication of mulberry has not been studied in detail. The number of species and varieties differs according to the authors since the entities classified as species by some taxonomists, are considered as varieties of a species by others. The considerable variation and

Keywords. DAMD; ISSR; Marker index; NJ tree; RAPD

[§]NBRI communication No. 542.

Abbreviations used: AFLP, Amplified fragment length polymorphism; DAMD, directed amplification of minisatellite DNA; ISSR, inter simple sequence repeat; NJ, neighbour-joining; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; SPAR, single primer amplification reaction; SSR, simple sequence repeat.

plasticity in morphological characters and sex expression of the species and the varieties as well as the natural cross-fertilization among most of the species and varieties has further resulted in a continuous character variation, making delimiting of plants into specific taxonomic groups difficult (Dandin 1998). There is thus a need for an internationally acceptable identification key for the varieties and species based on the existing genetic diversity.

Diversity analysis has been reported in case of mulberry genotypes for many different parameters (Rajan *et al* 1997; Fotadar and Dandin 1998; Tikader and Roy 2002) but less systematic work has been done on assessment of DNA profile-based diversity in mulberry. Recently random amplified polymorphic DNA (RAPD) or inter simple sequence repeat-polymerase chain reaction (ISSR-PCR) methods have been used for characterization of the Indian mulberry species and varieties (Vijayan and Chatterjee 2003; Awasthi *et al* 2004; Chatterjee *et al* 2004; Vijayan 2004; Vijayan *et al* 2004a,b). We have earlier reported RAPD and directed amplification of minisatellite DNA (DAMD) profile analysis of nine mulberry varieties (Bhattacharya and Ranade 2001) with the DAMD method being applied to mulberry diversity analysis for the first time to our knowledge. Amplified fragment length polymorphism (AFLP) analysis of the mulberry varieties has also been reported (Sharma *et al* 2000). Many of these studies have reported a wide genetic diversity in mulberry, based on the application of one or two methods at a time. Despite the number of studies, no information is available about the congruence of results by the different methods as well as about the usefulness of a given method over others. The wide range of diversity has also been interpreted as evidence for the naturalization of the mulberry varieties at places far from their origins, but other than the AFLP data of Sharma *et al* (2000) no other study has supported this naturalization.

We have studied diversity assessment in same set of mulberry varieties using three different PCR based single primer amplification reaction (SPAR) methods that are useful for genetic diversity studies in plants and collectively provide a comprehensive description of the nature and extent of the diversity. These three SPAR methods are routinely used in our work on diversity analysis in higher plants and include DAMD when a minisatellite core sequence is used singly as the primer (Heath *et al* 1993); ISSR-PCR when a simple sequence repeat (SSR) primer is used singly (Gupta *et al* 1994) and RAPD when the arbitrary sequence decamers are used as single primers (Williams *et al* 1990; Welsh and McClelland 1990).

In this paper, we compare the efficiencies of these three SPAR methods, individually as well as collectively, for diversity analysis of 27 mulberry varieties and we show that DAMD not only reveals the maximum polymorphism but also has the highest marker index amongst the three methods. This method may therefore, prove to be the best for analysing the mulberry germplasm. Further, we have observed only small differences in diversity amongst the exotic and indigenous groups of varieties in agreement with the suggested naturalization of the varieties.

2. Materials and methods

2.1 *Plant material*

The young non-senescing leaves were collected from plants of 27 mulberry varieties, from three different centres (table 1). In all cases, leaf tissue was stored frozen at – 70°C in powdered form after freezing at liquid nitrogen temperatures.

2.2 *Isolation of DNA*

Total plant DNA was isolated from the frozen tissue powder according to the method of de Kochko and Hamon (1990) with some modifications as described earlier (Bhattacharya and Ranade 2001).

2.3 *Minisatellite, SSR and RAPD primers*

Four minisatellite core sequence primers (M13 according to Lorenz *et al* 1995; 33·6, HBV and HVR according to Zhou *et al* 1997), and three SSR primers $(GAA)_6G$, $(CA)_8GC$ and $(ACTG)_4$ were custom synthesized from Bangalore Genei, Bangalore, India. The RAPD primers were procured from Qiagen Operon Technology Inc., Alameda, CA, USA.

2.4 *Amplification reactions*

The SPAR with minisatellite primers was carried out essentially according to Zhou *et al* (1997) for the primers 33·6, HBV and HVR(-) while for primer M13 the reactions were essentially according to Lorenz *et al* (1995) after first determining the optimum annealing temperature from the range 40°C to 55°C for each primer. The SPAR with SSR primers was carried out according to Gupta *et al* (1994). Here also, the optimum annealing temperature for each primer was first determined. SPAR for RAPD primers from kits B, F, G and H (Operon Technologies Inc., Alameda, California, USA) was carried out essentially following Williams *et al* (1990) after first optimizing most of the PCR parameters (data not shown). All amplification reactions were carried out in air thermal cycler (Model ATC1605, Idaho Technology,

Inc., USA) and the products were separated by electrophoresis (at constant current of 25mA) through 1·2 or 1·8% agarose gels (for DAMD and ISSR profiles) and 1% agarose gels for the RAPD reactions. All gels were made in 0·5X TBE buffer and the profiles after electrophoresis were visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining the gels with ethidium bromide. Only distinct and well-separated bands were included in the analysis.

2.5 *Data analysis*

Data (fragment sizes of all amplification products, estimated from the gel by comparison with standard molecular weight marker, *l* DNA double digested with *Hin*dIII and *Eco*RI) were scored as discrete variables, using '1' to indicate the presence and '0' to indicate the absence of a band. A pair wise matrix of distances between genotypes was determined cumulatively for all three methods using Jaccard coefficient (Jaccard 1901) in the FreeTree program (ver. 0·9·1·5; Pavlicek *et al* 1999). This distance matrix was used to compute a single neighbour-joining (NJ) tree after allowing a 500 replicate bootstrap test using the same program. The tree was viewed, annotated and printed using TreeView (ver. 1·6·5; Page 2001). The robustness of the SPAR methods was tested in each case with a suitable non-mulberry outgroup DNA included in the analysis, and in all cases outgroup was resolved as distinct from the mulberry DNAs (data not shown).

2.6 *Comparison of the different SPAR methods*

To determine the utility of each of the marker systems used, diversity index (DI), effective multiplex ratio (E) and marker index (MI) were calculated according to Powell *et al* (1996). The Mantel matrix correspondence test (Mantel 1967) was used to compare distance matrices for each SPAR method with the help of the program Mantel 2 (Liedloff 1999).

Table 1. The varieties of mulberry from which leaf tissue was collected for the present studies are listed. The accession numbers, where given, as well as, the variety names and origin are mostly from the passport data for those varieties, obtained from CSGRC, Hosur.

Sample No.	Tissue collected from	Accession No.	Variety name	Country/State
E1	CSGRC, Hosur	ME[a]-0078	Kairo roso	Japan
E2	CSGRC, Hosur	ME-0021	Kokuso-13	Japan
E4	CSGRC, Hosur	ME-0066	Kosen	Japan
E6	CSGRC, Hosur	ME-0098	Shimanochi	Japan
E7	CSGRC, Hosur	ME-0083	Canton china	China
E9	CSGRC, Hosur	ME-0033	Thailand male	Thailand
E11	CSGRC, Hosur	ME-0023	Calabresa	Paraguay
E13	CSGRC, Hosur	ME-0003	Moulai	Myanmar
E15	CSGRC, Hosur	ME-0084	Bogura-4	Bangladesh
E17	CSGRC, Hosur	ME-0026	PKS 248	Pakistan
I5	CSGRC, Hosur	MI[b]-0049	S-54	Karnataka
I7	CSGRC, Hosur	MI-0040	Acc. 112	Karnataka
I8	CSGRC, Hosur	MI-0022	Zangadud	Jammu and Kashmir
I11	CSGRC, Hosur	MI-0082	Acc. 106	Jammu and Kashmir
I12	CSGRC, Hosur	MI-0175	UP	Uttar Pradesh
I14	CSGRC, Hosur	MI-0007	Himachal Local	Himachal Pradesh
I15	CSGRC, Hosur	MI-0106	Heipanbi	Manipur
I17	CSGRC, Hosur	MI-0025	MR-2	Tamil Nadu
I19	CSGRC, Hosur	MI-0129	Matigara white	West Bengal
I20	CSGRC, Hosur	MI-0158	C-776	West Bengal
I22	CSGRC, Hosur	MI-0080	BC2-59	West Bengal
I23	CSGRC, Hosur	MI-0205	Berhampore-4	West Bengal
I25	CSGRC, Hosur	MI-0187	Tr-4	West Bengal
I26	CSGRC, Hosur NBRI, Lucknow	MI-0191	Tr-8	West Bengal
I27	Bharatidasan University, Trichi; NBRI, Lucknow	NA[c]	Tr-10	West Bengal
I28	CSGRC, Hosur	MI-0129	Tr-22	West Bengal
I29	CSGRC, Hosur	MI-0158	T-2	West Bengal

[a]Accession numbers with prefix ME are for the exotic varieties.
[b]Accession numbers with prefix MI are for the indigenous varieties.
[c]Accession number not available.

2.7 *Diversity assessment in groups of varieties*

For this the varieties were considered as groups of exotics (that included 10 varieties) and indigenous (17 varieties) based on whether the varieties were originally from countries other than India or from India respectively (table 1). The cumulative band data by all three SPAR methods for these two groups was analysed using the program POPGENE (ver. 1·31, Yeh *et al* 1997).

3. Results

3.1 *SPAR analysis and profile polymorphism*

In the present study, three SPAR methods, DAMD, ISSR and RAPD, were used to reveal genetic variability in 27 mulberry varieties. In all cases PCR conditions were carefully optimized for both the thermal cycler as well as the reaction components (data not shown). The three methods revealed discrete banding patterns for all the primers many of which revealed 100% polymorphism across all the varieties. The cumulative size ranges for the amplified fragments as well as the numbers of fragments amplified are given in table 2. The DAMD method resulted in more bands per primer as compared to the other methods. To determine the efficiency of the three methods, diversity index (DI) or heterozygosity index, marker index (MI) and effective multiplex ratio (E) were separately calculated in case of each method used. The DAMD revealed the highest DI (0·32), E (14·5) and MI (4·6) amongst the three methods (table 2). These values suggest that the DAMD method was relatively the best for assessing diversity in the mulberry varieties. The cumulative data for all three SPAR methods was used to compute pair wise distances, which ranged widely from 0·30 to 0·68 between pairs of varieties. The NJ tree after a 500 replicate bootstrap test of robustness is shown in figure 1. The NJ tree consists of at least three major clusters marked A, B and C with large parenthesis in figure 1. Each of these clusters included both exotic and indigenous varieties. However, varieties in cluster C are relatively more distant from those in the clusters A and B. Cluster A may be further considered in two subclusters A1, that includes separately the Japanese (E1, E2, E4, E6) and indigenous (I11, I12, I14, I15) varieties and subcluster A2 that included indigenous variety I17 along with three exotic varieties (E13, E15, E17). Cluster B is more interesting because it includes a single exotic variety E9 (from Thailand) along with six indigenous varieties (I19, I25–I29, all from West Bengal). Cluster C is another mixed cluster including Chinese and other exotic varieties along with remaining indigenous varieties. The NJ tree overall does not show any obvious correlation between geographical or genetic diversity amongst the varieties.

3.2 *Comparison of the exotic and indigenous groups of varieties*

The entire set of 27 varieties used in the present study was considered in two groups, exotic (10 varieties) and indigenous (17 varieties). The cumulative band data for all three methods was subjected to Nei and Shannon-Weaver estimates of genetic diversity and the determined values are given in table 3. These estimates are a robust measure of gene diversity when all bands are treated as loci with a maximum of two alleles possible and with an implicit assumption that the genotypes in a population or a group are in Hardy-Weinberg equilibrium. This assumption is important because the varieties that we have included in the present study are not derived from closed interbreeding populations and are distinct genotypes that may or may not have related lineages. This is also the reason why the diversity estimates are not supported by estimation of partitioning of the diversity within and amongst the two groups of varieties. The results indicate that though the exotic group of varieties had slightly higher values for both Nei and Shannon-Weaver estimates of diversity, than those for the indigenous varieties, the two groups of varieties were not so strongly diverged from each other.

4. Discussion

Mulberries can easily cross-pollinate and inter-species hybrids of mulberry have been reported. In order to assess genetic diversity in such plants, a three-way profiling approach was used. The DAMD and ISSR profiles reveal information about diversity of mulberry genomes in regions rich in repetitive sequences while the RAPD pro-

Table 2. The various SPAR methods and details of their results and analysis.

SPAR method	Number of primers used	Numbers of bands (cumulative total number of bands)	Size range of bands (bp)	Number of polymorphic bands per primer	Effective multiplex ratio (E)	Diversity index (DI)	Marker index (E × DI)
DAMD	4	12–18 (58)	340–1850	14·5	14·50	0·32	4·6
ISSR	3	8–16 (39)	260–1650	13	11·08	0·30	3·3
RAPD	20	7–21 (235)	240–2590	11·7	10·67	0·29	3·1

files collectively represent a much larger portion of the genome, if not actually the entire genome. Such an approach was expected to reveal a comprehensive pattern of genetic diversity in mulberry, which could then be used for characterization of the entire germplasm and possibly of individual varieties also. These SPAR methods have been used for diversity analysis in several plants such as mulberry, neem and papaya (Bhattacharya and Ranade 2001; Ranade and Farooqui 2002; Saxena *et al* 2005), blackgram (Ranade and Gopalkrishna 2004), Italian populations of *Asparagus acutifolius* L. (Sica *et al* 2005) and velvet bentgrass (Hollman *et al* 2005). In case of an endangered plant like *Neolitsea sericea* of family Lauraceae, the population structure has also been studied on the basis of SPAR profiles (Wang *et al* 2005).

4.1 *SPAR profile diversity in mulberry*

The SPAR methods resulted in discrete banding patterns in the present study and all three methods resulted in

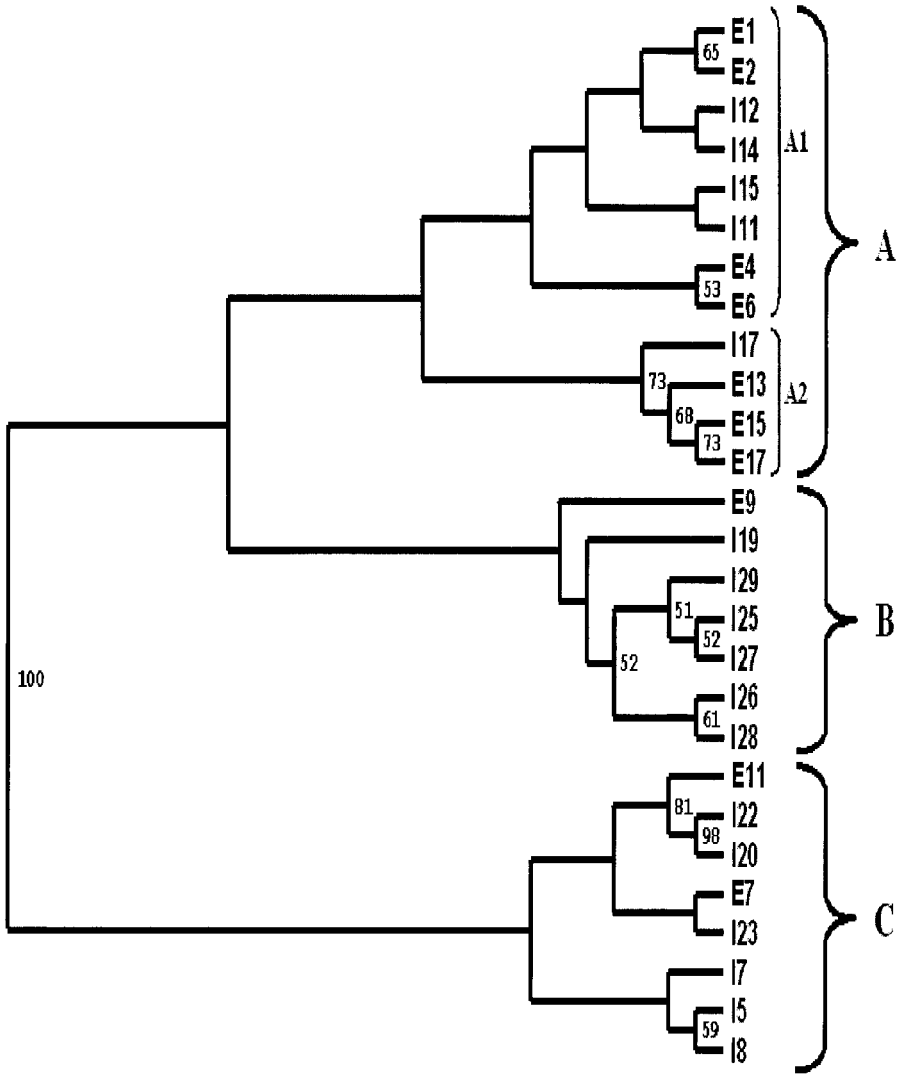

Figure 1. Cluster analysis of SPAR data in case of the mulberry varieties after a 500 replicate bootstrap analysis. The NJ tree was generated for cumulative band data by all three SPAR methods, DAMD, ISSR-PCR and RAPD. The variety names are abbreviated as in table 1, and are indicated to the right of the tree. The branch lengths are based on the distance values computed using NJ method and Jaccard coefficient in the program FreeTree. The numbers at the nodes in each tree are the bootstrap percent values (only values greater than or equal to 50 are shown) for the branches to the right of the node. The large parenthesis to the right labelled with A-C are the three major clusters while the two inner parenthesis labelled A1 and A2 are the two subclusters within cluster A.

polymorphic profiles. The DAMD method exhibited the highest percentage of polymorphic bands per assay as compared to that by the other two SPAR methods. The three SPAR methods individually resulted in the conclusion each time that the genetic diversity in mulberry was high since the range of pairwise distances was also high in each case (data not shown). A Mantel test (Leidloff 1999) of correlation analysis of the distance matrices was carried out and resulted in significant correlation ('*g*' standard variate > $P_{0\cdot005}$ value) in all cases. However, the highest correlation was between RAPD and ISSR-PCR distance matrices ($r = 0\cdot7236$) while the correlations between RAPD and ISSR distance matrices to that by DAMD were 0·4553 and 0·4827 respectively. This clearly indicates that relative distances of the varieties to each other were almost similarly estimated by both RAPD and ISSR-PCR methods. The three methods did not result in any congruence between pair wise distances and variety groups or even geographical origin of the varieties. So, in order to have a comprehensive pattern of genetic diversity in mulberry the cumulative data for all three SPAR methods was used to compute pair wise distances and the NJ tree. The distances (0·30 to 0·68) were in a wide range indicating thereby, that mulberry represents a genetically diverse population. This is possible because it is highly out-bred. Sharma *et al* (2000) also found a similar wide range in genetic distances (0·01 to 0·42) in mulberry by using AFLP method.

The tree obtained by the NJ method (figure 1) for the cumulative data did not show any significant correlation between the genetic divergence and the geographical distribution. The tree also did not reveal any clear separation of the exotic and indigenous varieties. The entire NJ tree indicates at least three major clusters (marked A, B and C with large parenthesis in figure 1) of the mulberry varieties. Cluster A is actually further divided into subclusters A1 and A2 (figure 1). Subcluster A1 includes separate groups of all the Japanese and some of the indigenous varieties indicating that these Indian and Japanese varieties are genetically distinct from each other while A2 subcluster includes a single indigenous variety along with three different exotic varieties from Myanmar, Bangladesh and Pakistan. Cluster B is interesting in having a single Thailand variety along with 6 of the West Bengal varieties. Presumably these West Bengal varieties have greater affinity to the variety from Thailand either by descent or as progenitors. Except variety I19, the other indigenous varieties are either triploid (I25–I28) or tetraploid (I29) and all of which are presumably derived from the ancestral variety(ies) that is related to but is not I19. Considering that the varieties have been in cultivation for long after their selection or introduction for the sericulture, it is possible that the genetic diversity in these varieties is derived from several putative parental lines that could have been involved as the varieties were developed or evolved. Yet we can also suggest that the three major clusters may represent at least three prominent genetic lineages of the mulberry varieties. A confirmation for this would, however, require an exhaustive genealogical survey of global mulberry accessions in much the same way that was recently done for barley (Jacob *et al* 2004; Blattner 2004) and a few other plants.

The exotic and indigenous varieties belonging to the same geographical region also did not show any significant grouping. This is similar to the results of a multivariate analysis using Mahalanobis D^2 statistic in case of 98 and 146 mulberry varieties from different geographic regions (Rajan *et al* 1997; Tikader and Roy 2002), as well as in other plants like *Hippophae rhamnoides* and native Americam maize (Bartish *et al* 1999; Moeller and Schaal 1999). According to Sharma *et al* (2000), the lack of any congruence between genetic and geographic variations in mulberry could be due to the fact that most of the mulberry genotypes are considered 'naturalized' due to their establishment, adaptation and persistence in areas distant from their origin. Our data too indicate that some of the exotic varieties, from distant places where sericulture is a relatively new industry, always grouped with varieties

Table 3. The proportion of polymorphic bands and mean index of diversity for the two groups of varieties based on cumulative data of all three SPAR methods.

Group (number of varieties)	Number of bands (cumulative data for all primers, all methods)		Mean Nei index[a] (H) ± SD	Mean Shannon index[a] (I) ± SD
	Total	Polymorphic		
Exotic (10)	332	266	0·2393 ± 0·1742	0·3699 ± 0·2414
Indigenous (17)	332	255	0·2262 ± 0·1837	0·3484 ± 0·2545

[a]Mean Nei index H (Nei 1973) and Mean Shannon index I (Shannon and Weaver 1949) were calculated using the program POPGENE ver. 1·31.

from the Southeast Asian countries. When the silk trade was going on between different nations, mulberry germplasm was taken from the countries of its origin and planted in the newer areas in Europe, Latin America and Africa. This can be seen in the NJ tree (figure 1) where in a cluster C, variety Calabresa (E11, table 1) from Paraguay occurs along with Indian varieties (I20 and I22) or in subcluster A2, the variety Moulai (E13) from Myanmar clusters with PKS 248 (E17) from Pakistan and Bogura-4 (E15) from Bangladesh and all these three exotic varieties are closest to the indigenous variety MR2 (I17). Probably the genotypes now being grown in Paraguay, Pakistan and Bangladesh actually came from the Indian or Myanmar region respectively.

4.2 *Efficiency of the different methods used to assess the genetic diversity in mulberry*

A comparative analysis of the three PCR-based methods used both in case of exotic and indigenous varieties of mulberry was carried out to assess individual methods. The DAMD method revealed the highest DI, E and MI values as compared to the other two methods (table 2). Though the distance values were different for each method the high degree of variation or divergence in mulberry shown by each method was as expected for an out-breeding species. It was interesting to notice that the distance data obtained from RAPD profiles did not correspond to those obtained from ISSR-PCR and DAMD. Such observations were also made in case of lentil (Sonnate and Pignone 2001) where the authors could not find any congruence between the results of RAPD and ISSR methods. Carvalho and Schaal (2001) also obtained different levels of polymorphism in cassava, where the SSR-primed markers showed less polymorphism than the RAPD markers. In their case also grouping of varieties from different geographical habitats varied between the RAPD and ISSR methods. From these results it is apparent that the different methods individually reveal information about distinct and different regions of the genome. However, we suggest that by combining different methods we may arrive at a better assessment or explanation for the diversity in plants. Though the present work has not shown specific grouping of the varieties based on geographical origins this is more likely a result specific for mulberry where the varieties have been established at places far distinct from the original native distribution. In contrast, in case of papaya (Saxena *et al* 2005), a similar SPAR analysis showed that though individual SPAR methods revealed non-congruent results, however, a cumulative analysis clearly resulted in a tree that correlated with the papaya pulp colour characteristic clearly showing the usefulness of a combined analysis of more than one SPAR method.

4.3 *Diversity of the exotic and indigenous mulberry varieties*

The Nei and Shannon-Weaver estimates of genetic diversity (table 3) revealed that the exotic group of varieties had the relatively greater diversity, though the actual values for the two groups are not so greatly differing from each other. This observation is very significant because it shows the two groups of varieties are not so largely diverged from each other as one would expect if the varieties are originating from geographically well separated regions and countries. In mulberry, there is perhaps the unique situation of an ancient dominant silk trade influencing the genetic relationships of mulberry varieties since many of the varieties are not native to the places where they are grown far from the sites of actual origin as has been outlined in case of *Morus alba* by Sharma *et al* (2000). In the Indian subcontinent, mulberry varieties are also maintained or propagated through stem-cuttings thereby “apparently fixing” variations amongst the varieties (Chatterjee *et al* 2004). Thus an ancient mixing of genotypes due to exchange of germplasm for promoting sericulture, putative hybridization between these and native plants and lack of dominant sexual reproduction mode for maintenance of germplasm could have resulted in the so called ‘naturalized’ varieties and this is supported by the observations first by Sharma *et al* (2000) and in the present study, where geographical origin alone can not distinguish the varieties.

The small differences in diversity estimates are probably because relative to the indigenous group, the exotic group of varieties did not include any derived varieties from breeding experiments or selection trials. The indigenous group of varieties on the other hand included several varieties from selection trials based on parameters of improved field performance as well as from breeding experiments, as for example the variety BC2-59 (I22) that is derived from a cross involving C-776 (I20) as one of the parents. This fact has also resulted in these two indigenous varieties exhibiting the least distance from each other (figure 1, cluster C). The different varieties have sufficient affinities between groups also. Supporting this conclusion the NJ tree clearly shows exotic and indigenous varieties in same sub-clusters even amongst the varieties of the primary sericulture regions such as India, Japan, China and Thailand. It would actually be very interesting to study the diversity amongst wild mulberry plants as well as amongst specific groups of local varieties in more than one geographical region to have a better understanding of the natural partition of genetic diversity

and the different genetic lineages that represent today's cultivated mulberry varieties.

5. Conclusions

In our study we found that DAMD was the method, which showed the highest polymorphism and the highest marker index. Thus, it appears that DAMD can be the method of choice for diversity analysis type of studies, in so far as polymorphism or marker index is the criterion. The data from three independent methods clearly showed that the mulberry genome has considerable diversity. The combined NJ tree for data obtained by all three SPAR methods supports the concept of 'naturalization' of varieties at locales distant from their actual origins, though for a stronger conclusion on these lines, a genealogical survey of mulberries with a multi-parameter and multi-method approach may be required, while to obtain a robust estimate of the range in diversity, DAMD alone is also adequate for mulberry.

Acknowledgements

Authors thank Director NBRI for support, Council of Scientific and Industrial Research, New Delhi for financial support as research studentship (SRF) to EB and Prof. M Vivekanandan, Bharatidasan University, Tiruchirapally, Tamil Nadu, for allowing collection of mulberry leaves from the plants maintained there.

References

Awasthi A K, Nagaraja G M, Naik G V, Sriramana K, Thangavelu K and Nagaraju J 2004 Genetic diversity and relationships in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays; *BMC Genet.* **5** 1 (http://www.biomedcentral.com/content/pdf/1471-2156-5-1.pdf)

Bartish I V, Jeppsson N and Nybom H 1999 Population genetic structure in the dioecious plant species *Hippophae rhamnoides* investigated by random amplified polymorphic DNA (RAPD) markers; *Mol. Ecol.* **8** 791–802

Bhattacharya E and Ranade S A 2001 RAPD and DAMD profile differences amongst mulberry varieties; *BMC Plant Biol.* **1** 3 (http://www.biomedcentral.com/content/pdf/1471-2229-1-3.pdf)

Blattner F R 2004 Phylogenetic analysis of *Hordeum* (Poaceae) as inferred by nuclear rDNA ITS sequences; *Mol. Phylogenet. Evol.* **33** 289–299

Carvalho L J C B and Schaal B A 2001 Assessing genetic diversity in the cassava (*Manihot esculenta* Crantz) germplasm collection in Brazil using PCR-based markers; *Euphytica* **120** 133–142

Chatterjee S N, Nagaraja G M, Srivastava P P and Naik G 2004 Morphological and molecular variation of *Morus laevigata* in India; *Genetica* **121** 133–143

Dandin S B 1998 Mulberry: A versatile biosource in the service of mankind; *Acta Sericologica Sinica* **24** 109–113

de Kochko A and Hamon S 1990 A rapid and efficient method for the isolation of restrictable DNA from plants of the genus *Abelmoschus*; *Plant Mol. Biol. Rep.* **8** 3–7

Fotadar R K and Dandin S B 1998 Genetic divergence in the mulberry; *Canye Kexue*. **24** 180–185

Gupta M, Chyi Y S, Romero-Severson J and Owen J L 1994 Amplification of DNA markers from evolutionarily diverse genomes using single primers of SSRs; *Theor. Appl. Genet.* **89** 998–1006

Heath D D, Iwana G K and Delvin R H 1993 PCR primed with VNTR core sequences yield species specific patterns and hypervariable probes; *Nucleic Acids Res.* **21** 5782–5785

Hollman A B, Stiera J C, Caslerc M D, Jungb G and Brilmand L A 2005 Identification of putative velvet Bentgrass clones using RAPD markers; *Crop Sci.* **45** 923–930

Jaccard P 1901 Etude comparative de la distribution florale dans une portion des Alpes et des Jura; *Bull. Soc. Vaudoise Sci. Nat.* **37** 547–579

Jakob S S, Meister A and Blattner F R 2004 The considerable genome size variation of *Hordeum* species (Poaceae) is linked to phylogeny, life form, ecology, and speciation rates; *Mol. Biol. Evol.* **21** 860–869

Liedloff A 1999 *Mantel Nonparametric Test Calculator for Windows, version 2x00* (http://www.sci.qut.edu.au/nrs/mantel.htm)

Lorenz M, Partensky F, Borner T and Hess W R 1995 Sequencing of RAPD fragments amplified from the genome of the prokaryote *Prochlorococcus marinus* (Prochlorophyta); *Biochem. Mol. Biol. Int.* **36** 705–713

Mantel N 1967 The detection of disease clustering and a generalized regression approach; *Cancer Res.* **27** 209–220

Moeller D A and Schaal B A 1999 Genetic relationships among Native American maize accessions of the Great Plains assessed by RAPDs; *Theor. Appl. Genet.* **99** 1061–1067

Nei M 1973 Analysis of gene diversity in subdivided populations; *Proc. Natl. Acad. Sci. USA* **70** 3321–3323

Page R D M 2001 *TreeView (Win32) ver. 1x6x5* (http://taxonomy.zoology.gla.ac.uk/rod/rod.html)

Pavlicek A, Hrda S and Flegr J 1999 FreeTree – Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrapping/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*; *Folia Biol. (Praha.)* **45** 97–99 (http://www.natur.cuni.cz/~flegr/freetree.htm)

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S V and Rafalski A 1996 The utility of RFLP, RAPD, AFLP and SSRP (microsatellite) markers for germplasm analysis; *Mol. Breed.* **2** 225–238

Rajan M V, Chaturvedi H K and Sarkar A 1997 Multivariate analysis as an aid to genotype selection for breeding in mulberry; *Indian J. Seric.* **36** 111–115

Ranade S A and Farooqui N 2002 Assessment of profile variations amongst provenances of neem using single-primer-amplification reaction (SPAR) methods; *Mol. Biol. Today* **3** 1–10 (http://www.molbio.net/v3/01/01.pdf)

Ranade R and Gopalakrishna T 2004 DNA polymorphism among blackgram (*Vigna mungo* L. Hepper) genotypes using inter-simple sequence repeat (ISSR) markers; *Plant Genet. Res.* **2** 37–42

Saxena S, Chandra R, Srivastava A P, Mishra M, Pathak R K and Ranade S A 2005 Analysis of genetic diversity among papaya cultivars using Single Primer Amplification Reaction (SPAR) methods; *J. Hortic. Sci. Biotech.* **80** 291–296

Shannon C E and Weaver W 1949 *The mathematical theory of communication* (Urbana: University of Illinois Press)

Sharma A, Sharma R and Machii H 2000 Assessment of genetic diversity in a *Morus* germplasm collection using fluorescence-based AFLP markers; *Theor. Appl. Genet.* **101** 1049–1055

Sica M, Gamba G, Montieri S, Gaudio L and Aceto S 2005 ISSR markers show differentiation among Italian populations of *Asparagus acutifolius* L.; *BMC Genet.* **6** 17 (http://www.biomedcentral.com/conte0nt/pdf/1471-2156-6-7.pdf)

Sonnate G and Pignone D 2001 Assessment of genetic variation in a collection of lentil using molecular tools; *Euphytica* **120** 301–307

Tikader A and Roy B N 2002 Genetic divergence in mulberry (*Morus* spp.); *Indian J. Genet.* **62** 52–54

Vijayan K 2004 Genetic relationships of Japanese and Indian mulberry (*Morus* spp.) genotypes revealed by DNA fingerprinting; *Plant. Syst. Evol.* **243** 221–232

Vijayan K and Chatterjee S N 2003 ISSR profiling of Indian cultivars of mulberry (*Morus* spp.) and its relevance to breeding programs; *Euphytica* **131** 53–63

Vijayan K, Awasthi A K, Srivastava P P and Saratchandra B 2004a Genetic analysis of Indian mulberry varieties through molecular markers; *Hereditas* **141** 8–14

Vijayan K, Srivastava P P and Awasthi A K 2004b Analysis of phylogenetic relationship among five mulberry (*Morus*) species using molecular markers; *Genome* **47** 439–448

Wang Z-S, An S-Q, Liu H, Leng X, Zheng J-W and Liu Y-H 2005 Genetic structure of the endangered plant *Neolitsea sericea* (Lauraceae) from the Zhoushan Archipelago using RAPD markers; *Ann. Bot.* **95** 305–313

Welsh J and McClelland M 1990 Fingerprinting genomes using PCR with arbitrary primers; *Nucleic. Acids Res.* **18** 7213–7218

Williams J G K, Kubelik A R, Livak K J, Rafalski J A and Tingey S V 1990 DNA polymorphisms amplified by arbitrary primers are useful as genetic markers; *Nucleic Acids Res.* **18** 6531–6535

Yeh F C, Yang R-C and Boyle T 1997 *POPGENE – A Microsoft Windows-based freeware for population genetic analysis, ver. 1x31 (32 bit)* (http://www.ualberta.ca/~fcyeh/)

Zhou Z, Bebeli P J, Somers D J and Gustafson J P 1997 Direct amplification of minisatellite-region DNA with VNTR core sequences in the genus *Oryza*; *Theor. Appl. Genet.* **95** 942–949

MS received 18 April 2005; accepted 29 September 2005

*e*Publication: 15 November 2005

Corresponding editor: DEEPAK PENTAL