

Research article

## Molecular distinction amongst varieties of Mulberry using RAPD and DAMD profiles

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### Abstract

**Background:** Mulberry trees are the most important host for rearing mulberry silkworms in sericulture. Improved varieties of mulberry tree have been developed through traditional breeding procedures. Not much work, however, has been carried out on the molecular characterization of these varieties. Random Amplified Polymorphic DNA (RAPD) and Directed Amplification of Minisatellite DNA (DAMD) methods based on Polymerase Chain Reaction are important tools to analyze genetic diversity of mulberries. These have been used to determine variation amongst nine varieties of *Morus* spp. maintained at Banthra Research Station of National Botanical Research Institute, Lucknow.

**Results and Discussion:** The varieties were analyzed using 23 arbitrary sequence decamer primers for RAPD, and 3 minisatellite core sequence primers for DAMD reactions. The RAPD and DAMD band data, (a total of 200 bands), were used to determine the pair wise distances according to Jaccard's algorithm. From these distance values Neighbour Joining (NJ) analyses were carried out separately for the RAPD and the DAMD data. The triploid varieties were found to be most similar to each other using RAPD analysis, while the varieties S13 and S34 were more similar using DAMD analysis. Nearly 85% of the RAPD bands and 91% of the DAMD bands were polymorphic across the nine varieties.

**Conclusions:** The mulberry varieties could be distinguished by their RAPD and DAMD profiles. As many as five RAPD primers and one DAMD primer generated profiles that can together differentiate all the nine varieties in terms of unique bands.

### Background

Mulberry (*Morus* spp.), is the most important crop plant in sericulture as the mulberry silkworms (*Bombyx mori* L.) are reared on them. Mulberries are a group of small trees or shrubs belonging to the family Moraceae, distributed in the temperate and subtropical regions in the northern hemisphere. In India six species are generally found,

namely, *M. alba* L. (2x, 2n = 28), *M. indica* L., *M. atropurpurea* Roxb., *M. nigra* L., *M. serrata* Roxb. (6x, 2n = 84) and *M. laevigata* Wall. (4x, 2n = 56) [1,2]. There is, however, some confusion over the species identification and according to Dandin [3] the first three species should be treated as synonyms or varieties of *M. alba* L. while the other three should be retained as distinct species. Some

species have also been introduced into India from China and Japan, mainly for rearing silkworms, during the past four decades.

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 [4]. Mulberry genotypes have been evaluated with respect to many parameters but very little work has been done on characterization of mulberry DNA. The genetic identities and relationships of mulberry plants including cultivars, diploids and polyploids have been determined using RAPD technology [5–7]. Xiang *et al.* [8] also applied RAPD technique in systematics of the genus *Morus* L. While these studies focused on variation among different species of *Morus* very little is known about the intra-species genetic variation existing in mulberry. Furthermore, no molecular work has been reported on the genetic diversity amongst the mulberry plants grown in India.

In recent years DNA-based markers allowing direct comparison of the genetic material of two individual plants have been used quite extensively. The RAPD technique [9,10], provides a convenient and rapid assessment of the differences in the genetic composition of the related individuals and has been employed in a large number of plants for the determination and assessment of genetic diversity. The other technique, Directed Amplification of Minisatellite DNA (DAMD) has been developed by Heath *et al.* [11] and reveals polymorphism due to minisatellites. Since this technique is carried out at higher PCR-stringencies, the patterns produced have a greater reproducibility than RAPD. In this paper we report the results from the application of both RAPD as well as DAMD PCR profiling, for the determination of differences amongst nine varieties of mulberry. These included triploids and selected diploid varieties of importance to moriculture.

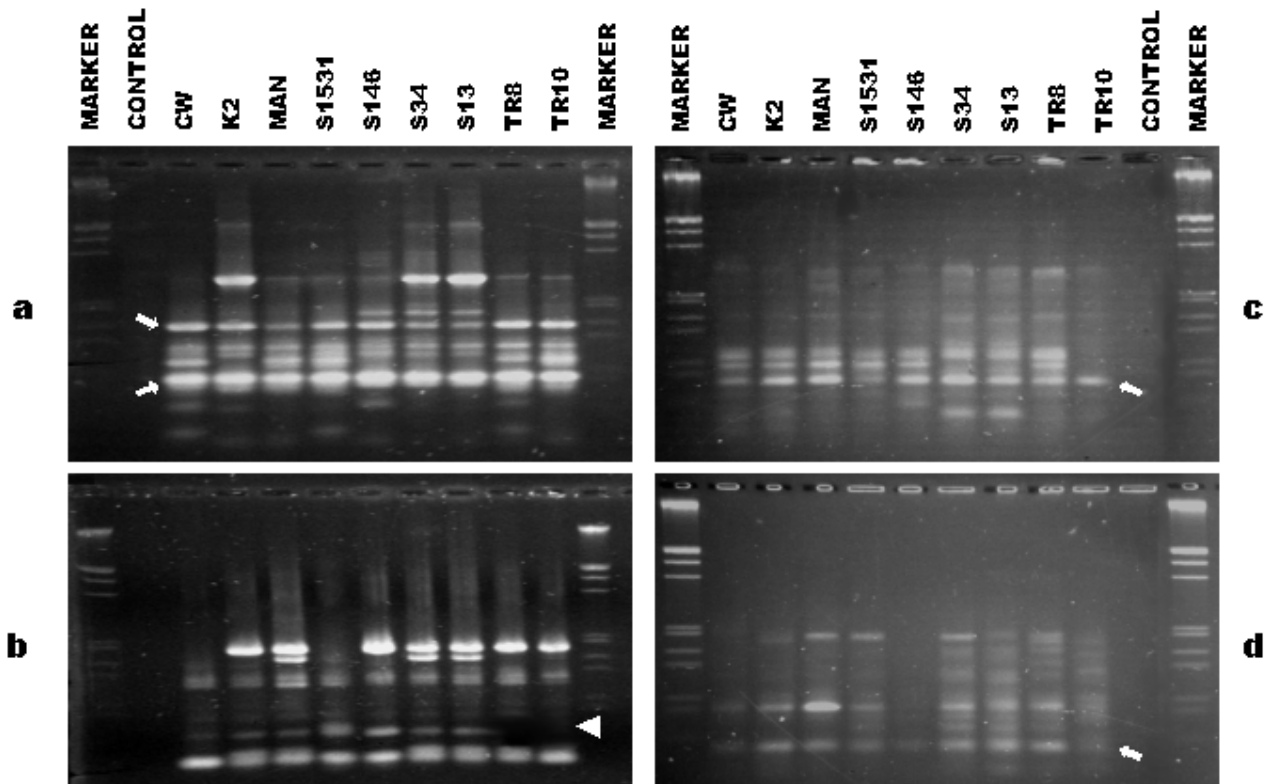
## Results

The DNAs isolated from nine varieties were found to be of high molecular weight as determined by agarose gel electrophoresis. These DNAs were used for the RAPD and DAMD reactions. RAPD conditions were optimized in a pilot experiment, varying concentrations of  $Mg^{2+}$  ions, primer and DNA (data not shown). Similarly, in case of three DAMD primers, annealing temperature was varied between 50°C and 55°C so as to determine the optimum temperature (data not shown). For  $Mg^{2+}$  titration, 0 mM, 1.5 mM, 2.5 mM, 3.5 mM and 4.5 mM  $MgCl_2$  were included in each reaction. Similarly 0, 5, 10, 15 and 20 pMoles of primers and 0, 12.5, 25, 50, 100 and 200 ng of DNA templates were also tested for amplification. Typically, in case of  $Mg^{2+}$  ions, a decrease in the concentration resulted in few or no bands due to the increase in stringency

of the reaction. An increase in primer concentration resulted in an increased number of bands. However, variation in DNA concentration, at least up to 200 ng of DNA used per reaction, did not result in differences in the profiles. On the basis of these results 1.5 mM  $MgCl_2$ , 10 pMoles primer and 50 ng of template DNA were considered to be optimal for generation of consistent RAPD profiles in mulberry and were, therefore, used in all the subsequent reactions with all the RAPD primers. In case of the DAMD PCR, annealing temperature of 50°C was found to be optimum for generating distinct profiles with all three primers.

In order to have a comprehensive idea of the variability amongst the varieties of mulberry, RAPD and DAMD analysis was carried out. For RAPD reactions, 40 arbitrary sequence decamers were used as primers. Of these, three primers did not give any profile at all, while 23 primers (Table 1) gave consistent (when the profiles with these primers were reproduced with at least three different preparations of the genomic DNAs) profiles. The RAPD data obtained with 23 primers and the DAMD data for three primers were considered separately for the analysis of the relationship amongst the different varieties. The profiles obtained with the RAPD primers OP-G16 and OP-G5, and the profiles with DAMD primers 33.6 and HVR in case of all nine mulberry DNAs are shown in Fig. 1 (a-d respectively). The fragments produced by these primers varied in size from 500–4200 bp. A total of 177 bands were scored in RAPD profiles (of which 26% or 44 bands were monomorphic) while 23 bands were scored in DAMD profiles (of which 9% or 2 bands were monomorphic). Bands were considered monomorphic since the same sized fragments were produced in all the varieties (as indicated by small arrows in Fig. 1a,c,d). The pair-wise distances were computed from the RAPD and DAMD band data and these values are given in Table 2. In RAPD method, maximum distance (0.44) was found between the varieties CW and S34, and the least distance (0.24) was between the triploid varieties TR8 and TR10. Similarly, in DAMD method, the maximum distance (0.78) was found between S146 and two varieties, namely, S13 and S34, while the least distance (0.13) was between S34 and S13.

The distance data were used to generate the NJ trees for both methods after carrying out 500 replicate bootstrap test. The NJ tree for RAPD method is given in Fig. 2a while that for the DAMD method is given in Fig. 2b. In both cases the varieties were grouped into clusters such that the topologies of the trees were broadly similar for some of the clusters of varieties. In the RAPD method, NJ tree revealed that the two triploid varieties TR8 and TR10 were the closest to each other while K2 was in general the most distant from all other varieties (Fig. 2a). When the RAPD data were subjected to a bootstrap test with 500 replicates,



**Figure 1**

RAPD and DAMD agarose gel electrophoresis profiles of mulberry DNAs. The profiles were obtained using primers OP-G16 (a), OP-G5 (b), 33.6 (c), and HVR (d). Lanes indicated by MARKER contain  $\lambda$  DNA double digested with *Hind*III and *Eco*RI as molecular weight marker. The variety names are abbreviated and marked at the top of the lanes and indicate the template DNA from the appropriate variety. The lane marked as CONTROL is a RAPD or DAMD reaction without any DNA template. The small white arrows in (a,c,d) indicate the monomorphic bands, while the small white arrow-head in (b) indicates the region in case of triploid varieties, TR8 and TR10, where a band that was present in all other varieties is absent.

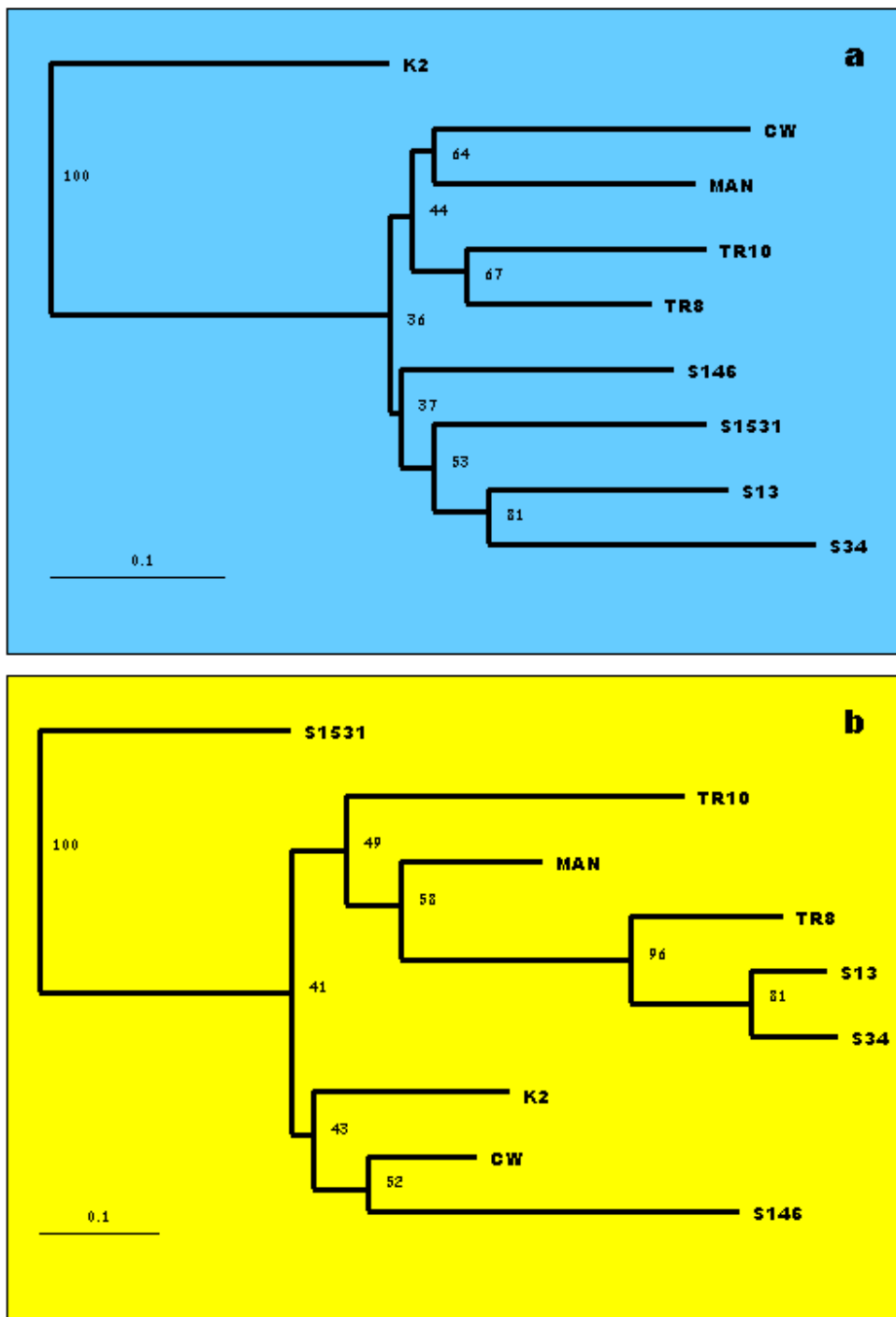
it resulted in 432 possible trees. The most frequently occurring tree amongst these grouped the indigenous variety (K2), exotic introduced varieties (CW and MAN), triploid varieties (TR8 and TR10) and the selection varieties (S13, S34, S146 and S1531) as separate clusters. The RAPD method was thus found to have a good resolution to distinguish between the varieties. The NJ tree generated from the DAMD data was different from that for the RAPD data. In the DAMD data, the varieties S13 and S34 were found to be the closest to each other while the triploid varieties grouped differently. The bootstrap test with 500 replicates resulted in 374 possible trees. The most frequently occurring tree amongst these clearly showed the above clustering of the varieties (Fig. 2b).

## Discussion

A large number of mulberry varieties are grown in several geographical regions of India both for their edible fruit

and for the silk industry. The identification of elite varieties or promising varieties of mulberry is important because it can ultimately have a direct effect on silk production and quality. One of the important ways by which these can be identified is to screen the mulberry germplasm for range and extent of genetic diversity present in it. In the recent times such an analysis of the germplasm has also been carried out using molecular techniques, including RAPD and other PCR-based screening techniques such as DAMD. With the help of RAPD, genetic variations have been detected, both, within and between species of plants [12–14]. DAMD-PCR has been applied for the fingerprinting of several plants including yeasts and fungi [15–18], wheat [19,20], rice [21], common bean [22] and neem [23].

In view of this, it was decided to employ the RAPD and DAMD techniques for the analysis of genetic variation in



**Figure 2**

Cluster analysis of RAPD and DAMD data in case of the mulberry varieties. The NJ trees by (a) RAPD method and (b) DAMD method are given as phylograms, respectively in blue and yellow panels. The numbers at the forks are the bootstrap percentage values while the scale in the bottom left corner of each panel is the distance scale based on the values in Table 2. The variety names are abbreviated and are indicated to the right of the trees.

**Table 1: The decamer sequences (all names beginning with OP) and the minisatellite core sequences used as primers in RAPD and DAMD reactions respectively, and the resulting bands in the profiles are listed.**

Primer	Sequence (5'-3')	Numbers of bands	
		Total	Monomorphic
OP-B1	GTTTCGCTCC	5	0
OP-B2	TGATCCCTGG	6	1
OP-B3	CATCCCCCTG	4	2
OP-B8	GTCCACACGG	8	1
OP-G2	GGCACTGAGG	12	2
OP-G3	GAGCCCTCCA	13	5
OP-G4	AGCGTGTCTG	4	2
OP-G5	CTGAGACGGA	6	2
OP-G8	TCACGTCCAC	9	0
OP-G10	AGGGCCGTCT	10	3
OP-G11	TGCCCGTCTG	9	2
OP-G12	CAGCTCACGA	11	4
OP-G13	CTCTCCGCCA	11	0
OP-G14	GGATGAGACC	4	1
OP-G15	ACTGGGACTC	5	2
OP-G16	AGCGTCCTCC	8	4
OP-G17	ACGACCGACA	5	3
OP-G18	GGCTCATGTG	8	2
OP-G19	GTCAGGGCAA	10	5
OP-AP2	TGGTCATCCC	6	0
OP-AP4	CTCTTGGGCT	8	2
OP-AP6	GTCACGTCTC	6	0
OP-API2	GTCTTACCCC	9	1
33.6 <sup>a</sup>	GGAGGTTTTCA	9	1
HBV <sup>b</sup>	GGTGTAGAGA-GAGGGGT	7	0
HVR <sup>c</sup>	CCTCCTC-CCTCCT	7	1

<sup>a</sup> – Core sequence of 33.6 minisatellite, [35]<sup>b</sup> – Core sequence of HBV minisatellite, [36]<sup>c</sup> – Core sequence of HVR minisatellite, [37]

case of an important plant like mulberry. The nine varieties used in this study included two indigenous triploids (TR8, TR10), four indigenous selections (S13, S34, S146 and S1531), the prominent indigenous variety Kanva-2 (K2) and two exotic varieties China White (CW) and Mandalay (MAN). The NJ tree after bootstrap test of RAPD data (Fig. 2a) clearly indicates the closeness of the varieties TR8 and TR10. Further, the varieties MAN and CW were also closer to both the triploid varieties than to the other varieties while all the selection varieties grouped together (Fig. 2a). On the other hand, the NJ tree after bootstrap test for the DAMD data revealed that the varieties S13, S34 and TR8 were least distant to each other while the four selection varieties were grouped differently relative to that in RAPD analysis. These groupings of the vari-

eties reflect the relative similarities of their genomic regions responsible for the RAPD or DAMD profiles. Of the two, the latter (DAMD) profiles were obtained with higher stringency PCR conditions than the RAPD profiles and therefore, the closeness of the varieties to each other in DAMD profiles is attributable to the similarities of their minisatellite sequence families.

The RAPD profiles revealed that the varieties could be distinguished broadly into groups of the selection varieties (S13, S34, S146, S1531), the triploids (TR8, TR10), the two exotic varieties (CW, MAN) and the indigenous variety K2. The DAMD profiles on the other hand resulted in a different clustering. The two techniques however, did not reveal any specific bias in the clustering of the varieties with reference to their geographical origins. This shows that the geographical divergence of the varieties is not reflected in their RAPD and DAMD profiles. It has been shown earlier also on the basis of multivariate analysis of nine different parameters that geographic diversity was not necessarily the direct cause of genetic diversity in mulberry [24].

RAPD profiles of the triploids were different from those of all the diploid varieties. Of these, however, the profiles in MAN and the triploid varieties TR8 and TR10 were relatively more similar. The distances of the two triploid varieties to MAN were 0.27 and 0.31, while amongst themselves, the distance was only 0.24 (Table 2, Fig. 2a). To the other diploid varieties the distances of the triploids were in the range 0.28 to 0.41. The number of bands found in the triploids was not particularly large as compared to that in the diploids even though they had one additional set of chromosomes. On the contrary, in case of primer OP-G5 it was observed that the triploid variety DNAs specifically lacked a band that was present in all other diploid varieties (as indicated by a small arrowhead in Fig. 1b). In case of sugarcane also, Harvey and Botha [25] did not observe any greater number of RAPD bands in polyploids relative to the diploids. In the case of the four indigenous selections, the clustering of the varieties by both methods indicates that S34 and S13 are most similar to each other (least distance 0.13 by DAMD method). The varieties CW and K2 seemed to be more distant from the other varieties and were clustered differently relative to them by both the methods (RAPD and DAMD).

## Conclusions

The overall extent of polymorphism was very high and the RAPD and DAMD data were useful in distinguishing between the nine varieties, despite including apparently diverse elements such as triploids and exotic varieties. As many as five RAPD primers and one DAMD primer generated profiles that can together differentiate all the nine varieties in terms of unique bands. These results are

**Table 2: Matrix of Jaccard's distances amongst pairs of varieties (names abbreviated). The figures in cells below the empty diagonal cells are the distances for RAPD band data while those in the cells above the empty diagonal cells are for the DAMD band data.**

	CW	K2	MAN	S1531	S146	S34	S13	TR8	TR10
<b>cw</b>		0.25	0.40	0.37	0.40	0.67	0.67	0.59	0.42
<b>K2</b>	<b>0.38</b>		0.31	0.39	0.54	0.65	0.58	0.58	0.53
<b>MAN</b>	<b>0.32</b>	<b>0.37</b>		0.40	0.63	0.50	0.42	0.42	0.44
<b>S1531</b>	<b>0.40</b>	<b>0.39</b>	<b>0.34</b>		0.54	0.59	0.59	0.59	0.54
<b>S146</b>	<b>0.34</b>	<b>0.36</b>	<b>0.34</b>	<b>0.32</b>		0.78	0.78	0.71	0.69
<b>S34</b>	<b>0.44</b>	<b>0.43</b>	<b>0.42</b>	<b>0.39</b>	<b>0.39</b>		0.13	0.24	0.68
<b>S13</b>	<b>0.42</b>	<b>0.34</b>	<b>0.37</b>	<b>0.30</b>	<b>0.34</b>	<b>0.31</b>		0.33	0.68
<b>TR8</b>	<b>0.36</b>	<b>0.36</b>	<b>0.27</b>	<b>0.28</b>	<b>0.30</b>	<b>0.36</b>	<b>0.35</b>		0.61
<b>TR10</b>	<b>0.35</b>	<b>0.35</b>	<b>0.31</b>	<b>0.37</b>	<b>0.35</b>	<b>0.41</b>	<b>0.37</b>	<b>0.24</b>	

consistent with the fact that mulberry is known to have highly heterozygous varieties with large numbers of natural hybrids between unisexual mulberry parents [26]. Supporting this, genetic divergence amongst as many as 58 varieties of mulberry was found to be high as determined by D<sup>2</sup> statistics of multivariate analyses [27]. The greatest distance (0.78) and the smallest distance (0.13) observed by the two methods in the present studies (Table 2) indicated that the varieties are between 22 and 87% similar. The two methods individually revealed that 85% (RAPD) and 91% (DAMD) bands were polymorphic. For a plant with extensive natural hybridization amongst varieties and the reportedly high genetic divergence, these values are thus indicative of the wide range of diversity. An alternative interpretation is that mulberry has only recently split from its ancestral taxonomic group. The present data on only nine varieties are, however, inadequate to really shed light on these two possibilities. Nonetheless, an important outcome of this study is the ability to distinguish between diverse varieties of mulberry using only a small number of RAPD and DAMD primers. Clearly, there is considerable scope for applying these techniques on a larger scale for the analysis of the entire mulberry germplasm.

## Material and methods

### Plant material

Mulberry plants of nine different varieties, namely, China White (CW), Kanwa2 (K2), Mandalay (MAN), S1531, S146, S34, S13, Triploid-8 (TR8) and Triploid-10 (TR10) were selected from amongst those being maintained at the Biomass Research Centre of NBRI at Banthra, on the outskirts of Lucknow.

### Isolation of DNA

Total plant DNA was isolated from fresh young leaves according to the method of de Kochko and Hamon [28]

with some modifications [29]. At least 3 independent DNA preparations were made from leaf tissues collected from each plant. The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel as well as by fluorometry (DyNA Quant 200, Pharmacia) using Hoechst 33258 as the fluorochrome.

### Polymerase Chain Reaction using RAPD primers

Decamers from kits B, F, G and AP (Operon Technologies Inc., Alameda, California, USA) were used as primers. DNA was amplified essentially following Williams *et al.* [9]. Initially a pilot experiment was carried out varying primer, template DNA and Mg<sup>2+</sup> ion concentrations. The final amplification reactions contained 10 mM TAPS (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 0.2 mM each dNTP, 10 pmoles primer, 0.6 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 50 ng mulberry DNA template in a 25 µl reaction volume. The reaction was cycled 44 times at 94°C for 1 min, 35°C for 1.5 min and 72°C for 1.5 min in a thermal cycler (Robocycler 40, Stratagene GmbH, Germany). Additionally a final extension cycle allowed incubation for 5 min at 72°C. Amplification products were separated by electrophoresis (at constant current of 25 mA) through 1.0% agarose gels in 0.5X TBE buffer, visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining with ethidium bromide.

### Polymerase Chain Reaction using Minisatellite core sequence primers for Directed Amplification of Minisatellite DNA

DAMD-PCR was carried out essentially according to Zhou *et al.* [21]. This technique involves the use of minisatellite core sequence as a primer, singly, in the amplification reactions. Other workers have also employed DAMD-PCR technique for determining both inter- as well as intra-species variations [15–23]. The optimum annealing

temperature was determined by carrying out DAMD-PCR at two different annealing temperatures, 50°C and 55°C. The PCR parameters were as follows: First cycle of incubation at 95°C for 2 min followed by 35 cycles of incubation at 95°C for 2 min, at annealing temperature for 1 min and at 72°C for 1.5 min. The final cycle allowed an additional incubation at 72°C for 5 min. The reactions contained 100 ng template DNA, 40 pmoles of primer, 2.5 mM Mg<sup>2+</sup> ions, 200 µM each dNTP, 0.3 U *Taq* DNA polymerase in 1× assay buffer in a final volume of 25 µl. The PCR products were separated by electrophoresis through 1% agarose gels and the profiles were analyzed as described for the RAPD profiles.

### Data Analysis

Data (fragment sizes of all amplification products, estimated from the gel by comparison with standard molecular weight marker, λ DNA double digested with *Hind*III and *Eco*RI) were scored as discrete variables, using "1" to indicate presence and "0" to indicate absence of a band. A pair wise matrix of distances between genotypes was separately determined for the RAPD (23 primers) and DAMD (3 primers) data using Jaccard's algorithm [30] in the RAPDistance package [31]. From these matrices, the NJ trees were generated according to Saitou and Nei [32], using the packages FreeTree [33] and TreeView [34].

### Abbreviations

DAMD – Directed Amplification of Minisatellite DNA, NJ – Neighbor Joining; RAPD – Random Amplified Polymorphic DNA.

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