International Journal of Plant Breeding ©2009 Global Science Books

Genetic Analysis of Diploid and Colchi-Tetraploid Mulberry (*Morus indica* and *Morus alba*) by Molecular and Morphological Markers

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ABSTRACT

This study attempts a comparative assessment of morphogenetic changes in mulberry due to colchicine treatment and doubling of chromosome complement. Out of the 10 morphological (qualitative) traits studied, eight showed changes in the diploid in comparison to its tetraploid in one or more traits. The two traits (lobation and leaf apex) remained unchanged in all the varieties irrespective of the treatment with colchicine and doubling of chromosome number. Although the SPAD (Soil Plant Analytical Device) value increased in many tetraploids in comparison to its diploids, S-13 and S-34 showed a negative correlation for SPAD values and thereby increase in ploidy levels. However, the SPAD value was significantly correlated with increased ploidy in the case of K-2 and S-36. Stomatal frequency was negatively correlated with higher ploidy, except in the case of K-2 and S-13. Among the 45 primers utilized for DNA fingerprinting, 35 showed at least one marker variation among one or more pairs of diploid mulberry varieties with respect to their colchi-tetraploid derivatives. Sequence analysis of the diploids and its tetraploids showed variation in the noncoding region of the genome. The result of the present investigation reveals that colchicine is capable of inducing ploidy, thus interfering at the morphological and molecular levels. It also demonstrates that RAPD markers are informative in divulging genetic variations taking place in the genome.

Keywords: colchiploids, genetic variation, mulberry improvement, polyploidy, RAPD markers

INTRODUCTION

Mulberry is one of the most widely distributed perennial tree species in the genus *Morus*, which is comprised of nearly 68 species occurring worldwide (Sanjappa 1989). Development of a superior cultivable hybrid is important to improve the productivity of mulberry leaf yield. Conventional breeding methods were utilized for the production of high-yielding mulberry varieties. There is a need to evolve region-specific varieties catering for the requirement of different agro-climatic and soil conditions in India. The mulberry varieties K-2 and RFS-135 developed through conventional methods were popular until the early 1990's and were suitable for irrigated and rain-fed areas of southern India (Susheelamma *et al*. 1990). Two drought-tolerant varieties, S-13 and S-34, were cultivated in dry regions. Among the new varieties; S-36 was nutritionally superior and used for silkworm rearing. The recently evolved hybrid variety V-1 has superior agronomic attributes with moderate resistance to 'leaf spot' and 'tukra' diseases. However, changing agro-ecological conditions and the region specific requirements insist on the development of new improved varieties.

In mulberry, natural polyploids like tetraploid (Janaki Ammal 1948; Datta 1954), hexaploid (Janaki Ammal 1948; Seki 1951) and even dodecasoploids (Basavaiah 1989; Dandin and Basavaiya 1995) have been reported. High ploidy levels in mulberry have been associated with undesirable agronomic traits and are seldom suitable for silkworm rearing. Conversely, triploid mulberry varieties have been found to possess desirable economic characteristics compared to their diploids. Generally, triploid mulberry varieties have thick, dark-green, succulent leaves and are nutritionally superior for rearing silkworms (Das and Prasad 1973; Sarkar 1993).

Conventionally, crossing tetraploid with a diploid variety produces triploids (Dwivedi *et al*. 1986). Therefore triploid breeding is normally preceded by the development of tetraploid parents. The alkaloid, colchicine is a potent inducer of chromosome doubling by inhibiting the formation of spindle fibers and thus arrests mitosis at anaphase. Chaicharoen *et al*. (1995) suggest that colchicine-induced tetraploid mulberry possesses *gigas* characteristics and several morphogenetic changes, in comparison with their diploid progenitors. The primary objective of this study was to assess the morphogenetic changes that accompany the induction of tetraploidy by colchicine in six different varieties of mulberry using morphological and molecular markers.

Recent advances in molecular biology provide a convenient and rapid assessment of the genetic composition of the related individuals using DNA sequence data (Zhao and Pan 2002; Zhao *et al*. 2005; Ravi *et al*. 2006) Molecular comparison and linking of physiological traits with molecular data is being practiced in the improvement of mulberry breeding (Vijayan *et al*. 2006). Molecular markers such as RAPD serve as dominant marker systems in identifying genetic and nuclear genomic variations in mulberry (Arvind 2004; Vijayan *et al*. 2004; Prasanta 2008) and other commercial crop varieties like rye grass (Castro *et al*. 2003) and wheat (Aliyev *et al*. 2007). Applications of PCR-based markers are used successfully for the evaluation of plant genetic resources in mulberry genotypes and are utilized for introgressive breeding strategies and genetic improvement. Ploidy induction serves as a tool to improve the existing genetic resources to express superior traits. The present study was an attempt to analyze the morphological and molecular variation induced by the mutant colchicine in mulberry variants.

Received: 1 October, 2008. Accepted: 15 May, 2009.

Original Research Paper

MATERIALS AND METHODS

Plant materials

Six diploid (2n=28) mulberry cultivars viz., RFS-135, V-1, K-2, S-30, S-34 and S-36 along with their tetraploid counterparts were utilized in the study. These plants were maintained in the mulberry experimental plot of the Central Sericultural Research and Training Institute, Mysore, under standard package of growth practices recommended by the Institute.

Colchicine treatment

Cuttings from the diploid plants were potted for acclimatization and allowed to establish. The apical buds of the saplings were selected at the green bud stage where the active cell division takes place. The buds were treated with 1% colchicine (Himedia Pvt., India) for three consecutive days. After the treatment period, the buds were allowed to sprout and the mature leaves were used for morphological variation analysis.

Morphological analysis

A total of 10 qualitative and three quantitative morphological traits were used to make a comparative assessment of morphology and genetic variation between diploid and tetraploid mulberry varieties. The qualitative morphological traits namely, branching pattern, phyllotaxy, lobation and leaf characteristics like color, texture, shape, margin, apex, base, and glossiness were analyzed by visually and microscopic examinations based on the consensus assessment of three replicated individual plants.

The quantitative parameters like chlorophyll content, internodal distance, and stomatal frequency were recorded as an average from three replications per variety. For stomatal analysis, three replications of 5th, 6th and 7th leaves from three-month old plants of all the accessions were collected and waxed on the lower surface of the leaf. The stomatal impressions were peeled off and the frequency was determined. Nine microscopic field observations were made for each accession. Stomatal frequency per mm^2 was determined by one-way ANOVA and Pearson's correlation strategy. The total chlorophyll content of the leaves was analyzed using the SPAD-502 chlorophyll meter (Konica Minolta). An average of ten fields from 5th, 6th and 7th leaves of a healthy plant was considered for the analysis. The correlation of chlorophyll values between diploid and tetraploid varieties was analyzed using the statistical package SPSS 13.0 and the significance was calculated.

DNA isolation

Fresh tender leaves from the diploid and colchicines-induced tetraploid mulberry varieties were collected for extraction of high molecular weight genomic DNA using Nucleon Phytopure Kit (Amersham Biosciences, UK) as per the manufacturer's instruction with suitable modification. The quality and quantity of DNA extracted was assessed using UV-Vis spectrophotometer as well as on 0.8% agarose gel (Amersham Biosciences, UK) after staining with 0.5 µg/ml ethidium bromide solution. The DNA stock solutions were diluted to a uniform concentration of 10 ng/µl for PCR amplification.

Selection of RAPD primers

The RAPD primers were selected by screening 50 random primers which shows highly reproducible polymorphic profiles among a set of diploid and its colchi-tetraploid mulberry genotypes. The random primers were obtained from Operon Technologies Inc., Almeda, USA.

RAPD-PCR amplification

PCR reactions were performed according to the protocol of Williams *et al.* (1990). The PCR amplification was carried in 0.2 ml DNase free PCR tubes in PTC-200 DNA engine (MJ Research, U.S.A.) with 20 µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 µM primer, 0.1 mM of each dNTPs, 0.5 U of *Taq* DNA polymerase (Genei, Bangalore) and 20 ng of template DNA. Amplification reactions were carried out by the following cycle profiles: initial denaturing cycle at 93°C for 2 min followed by 40 cycles at 93°C for 1 min, 35°C for 1 min, 72°C for 2 min and a final 7 min extension at 72°C. Markers amplified by the PCR reaction were electrophoresed in 1.5% agarose gel (Sambrook *et al.* 1989) in 1X TAE stained in ethidium bromide and the gel image was recorded using a gel documentation system (Syngene, UK).

Data analysis

Well-resolved and consistently reproducible markers in the range of 300-3000 bp generated by RAPD amplification were scored as "1" for the presence and "0" for the absence. Markers with the same migration distance were considered homologous. A pair-wise similarity matrix was calculated (Nei and Li 1979). A dendrogram was constructed based on the similarity matrix data set by applying un-weighted pair group method of arithmetic averages (UPGMA) using NTSYS 2.02i statistical software (Rohlf 1998).

Analysis of morphological parameters were done with SPSS 13.0 statistical package (SPSS Inc., Chicago, IL; http://www.spss.com) with P <0.05 considered significant. The data for morphological parameters were subjected one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). Pearson's correlation was used to analyze the relation between the morphological parameters and ploidy level. Each treatment consisted of three replications.

Sequence analysis

The mulberry sequence data was analyzed with BLAST (www.ncbi.nlm.nih.gov/blast, National Centre for Biotechnology Information) and the multiple sequence alignment study was conducted using an online EMBL tool, Kalign (Lassman and Sonnhammer 2005).

RESULTS

Variation in morphological characters

The list of diploid mulberry cultivars which were utilized for the development of auto-tetraploids by colchicine treatment along with their passport information is provided in **Table 1**. The study involved assessment of some of the important morphological parameters (**Fig. 1**) in diploid mulberry varieties and the impact in response to chromosome doubling (i.e., in colchi-tetraploids). Out of 10 morphological (qualitative) traits studied, eight showed changes in the diploid when compared to its tetraploid in one or more cases. Two characters (lobation and leaf apex) remained unchanged in all varieties irrespective of the treatment with colchicine and doubling of chromosome number. A comparative characterization of diploid mulberry cultivars and their colchi-tetraploid derivatives is provided in **Table 2**. On doubling of chromosome complement in the tetraploid, mulberry tends to show dark-green leaves in comparison with diploids, with the exception of RFS-135. The tetraploid RFS-135 had a light-green lamina in comparison to

Fig. 1 Leaf morphology of diploid and tetraploid mulberry varieties.

Table 1 List of diploid mulberry cultivars used in development of auto-tetraploidy along with passport data.

Cultivar	Species status	Origin	Sex	National Acc. №
RFS-135	*M. indica*	OPH of K-2	Male	IC-313694
V-1	*M. alba*	S-30 x Berhampore C-776	Male	--
S-36	*M. indica*	Mutant – EMS treatment of Berhampore Local	Female	IC-313678
K-2	*M. alba*	OPH of Mysore Local	Female	IC-313679
S-13	*M. indica*	OPH of K-2	Male	IC-313971
S-34	*M. indica*	S-30 x Berhampore C-776	Male	IC-313779

Table 2 Comparative morphological characteristics of diploid mulberry cultivars and their colchi-tetraploid derivatives.

Characters	RFS-135 (2X)	RFS-135 (4X)	V-1 (2X)	V-1 (4X)	S-36 (2X)	S-36 (4X)	K-2 (2X)	K-2 (4X)	S-13 (2X)	S-13 (4X)	S-34 (2X)	S-34 (4X)
Branch pattern	Semierect	Semierect	Erect	Erect	Semi erect	Spreading	Semi erect	Semi erect	Erect	Erect	Erect	Erect
Phyllotaxy	2/5	2/5	2/5	1/2	2/5	1/3	1/2	1/3	2/5	1/3	1/3	2/5
Lobation	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed	Unlobed
Leaf colour	Green	Light green	Dark green	Dark green	Light green	Green	Light green	Dark green	Green	Dark green	Dark green	Dark green
Leaf texture	Coriaceous	Coriaceous	Coriaceous	Coriaceous	Coriaceous	Coriaceous	Chartaceous	Coriaceous	Chartaceous	Coriaceous	Chartaceous	Coriaceous
Leaf shape	Ovate	Narrow ovate	Narrow ovate	Ovate	Wide ovate	Wide ovate	Ovate	Wide ovate	Narrow ovate	Wide ovate	Wide ovate	Wide ovate
Leaf margin	Serrate	Serrate	Serrate	Serrate	Crenate	Crenate	Crenate	Dentate	Serrate	Dentate	Serrate	Serrate
Leaf apex	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate	Acuminate
Leaf base	Cordate	Truncate	Truncate	Truncate	Cordate	Cordate	Cordate	Cordate	Truncate	Truncate	Cordate	Truncate
Leaf glossiness	Non glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Non glossy	Glossy	Non glossy	Non glossy	Non glossy	Non glossy

Table 3 ANOVA-Pearson correlation among diploid and tetraploid mulberry varieties.

Parameters	Varieties	Ploidy status	Mean± SE	F- value between groups	Correlation
SPAD	RFS-135	2x	31.46 ± 2.01	6.2985**	0.782047-NS
		4x	37.06 ± 0.95		
	K-2	2x	30.86 ± 0.87	32.7045**	0.943939**
		4x	36.46 ± 0.44		
	S-13	2x	38.2 ± 1.65	12.1969**	-0.86778**
		4x	32.16 ± 0.50		
	S-34	2x	40.36 ± 0.37	50**	-0.96225*
		4x	37.03 ± 0.28		
	S-36	2x	30.53 ± 0.95	22.8979**	0.922654*
		4x	35.6 ± 0.46		
	V-1	2x	30.9 ± 2.15	0.0893 NS	0.147806 NS
		4x	31.8 ± 2.10		
Internodal distance	RFS-135	2x	3.78 ± 0.27	0.8819 NS	-0.42504 NS
		4x	3.26 ± 0.48		
	K-2	2x	3.78 ± 0.27	3.6457**	-0.69053 NS
		4x	3.22 ± 0.11		
	S-13	2x	3.02 ± 0.17	1.5066 NS	0.523075 NS
		4x	3.53 ± 0.38		
	S-34	2x	2.58 ± 0.13	3.2933**	0.671977 NS
		4x	3.44 ± 0.45		
	S-36	2x	3.65 ± 0.31	0.9837 NS	-0.4443 NS
		4x	3.25 ± 0.25		
	V-1	2x	4.52 ± 0.23	0.3478 NS	-0.28284 NS
		4x	4.20 ± 0.48		
Stomatal frequency	RFS-135	2x	26.6 ± 1.12	176.022**	-0.978**
		4x	8.8 ± 0.73		
	K-2	2x	11.4 ± 1.03	1.091 NS	0.346 NS
		4x	12.6 ± 0.51		
	S-13	2x	18.4 ± 2.27	2.149*	-0.46014 NS
		4x	14.6 ± 1.25		
	S-34	2x	21.6 ± 1.21	32.021**	-0.894**
		4x	13.8 ± 0.66		
	S-36	2x	20.4 ± 0.98	18.964**	-0.839**
		4x	11.8 ± 1.71		
	V-1	2x	15.6 ± 0.81	15.432**	-0.812**
		4x	10.6 ± 0.98		

** Highly significant
* Correlation is significant at the (0.05) level.
** Correlation is significant at the (0.01) level.

the diploid counterpart. Subtle morphological variations can be observed, particularly in leaf characteristics on chromosome doubling.

Table 3 provides the details of ANOVA-Pearson correlation analysis of SPAD chlorophyll value, inter-nodal distance and stomatal frequency among the diploid, and its colchi-tetraploid varieties. Although the SPAD value increased in many tetraploids in comparison to its diploid counterparts, S-13 and S-34 showed a negative correlation for SPAD and increase in ploidy level. However, the SPAD

Table 4 Analysis of diploid and tetraploid mulberry genotypes.

Ploidy status	Varieties	Stomatal frequency	Internodal distance	SPAD chlorophyll content
2X	RFS-135	26.60 ± 1.12 a	3.79 ± 0.276 ab	31.47 ± 2.02 b
	K-2	11.40 ± 1.03 d	3.79 ± 0.28 ab	30.87 ± 0.87 b
	S-13	18.40 ± 2.27 bc	3.02 ± 0.17 bc	38.20 ± 1.65 a
	S-34	21.60 ± 1.20 b	2.59 ± 0.14 c	40.37 ± 0.38 a
	S-36	20.40 ± 0.98 b	3.65 ± 0.32 b	30.53 ± 0.95 b
	V-1	15.60 ± 0.81 c	4.52 ± 0.24 a	30.90 ± 2.15 b
F value		15.473**	7.606**	8.664**
4X	RFS-135	8.80 ± 0.73 c	3.27 ± 0.48	37.07 ± 0.95 a
	K-2	12.60 ± 0.51 ab	3.22 ± 0.11	36.47 ± 0.44 a
	S-13	14.60 ± 1.25 a	3.54 ± 0.39	32.16 ± 0.50 b
	S-34	13.80 ± 0.66 ab	3.45 ± 0.45	37.03 ± 0.28 a
	S-36	11.80 ± 1.71 abc	3.25 ± 0.25	35.60 ± 0.46 a
	V-1	10.60 ± 0.98 bc	4.21 ± 0.48	31.80 ± 2.11 b
F value		4.047**	NS	5.746**

** Significant at 1%; * Significant at 5%; NS- Non significant according to DMRT.

value was significantly correlated with increased ploidy in K-2 and S-36. In case of the inter-nodal distance there was no significant correlation with the increased ploidy level in mulberry cultivars. Stomatal frequency was negatively correlated with higher ploidy, except for K-2 and S-13. DMRT (**Table 4**) exhibited varying results for SPAD chlorophyll content, internodal distance and stomatal frequency within the diploid and tetraploid varieties. All the parameters were highly significant among the diploid genotypes. In tetraploids, chlorophyll content and stomatal frequency were highly significant among the varieties while the internodal distance was not.

RAPD marker variability

Genetic variation in DNA due to colchicine treatment and doubling of chromosome was evaluated by PCR amplification and comparison of both diploid and tetraploid genomes, simultaneously. A total of 50 arbitrary random primers were screened and 45 polymorphic primers were utilized for the study to develop informative DNA fingerprints. The PCR amplification revealed a total of 368 markers of which 294 were polymorphic (79.89%). As many as 11 primers showed 100% polymorphism. Among the 45 primers utilized for DNA fingerprinting, 35 primers showed at least one marker variation among one or more pair/s of diploid mulberry varieties with respect to their colchi-tetraploid derivatives.

Table 5 DNA marker variation among diploid and its colchi-tetraploid mulberry varieties.

Primer	Diploid/colchi-tetraploid mulberry cultivars	PCR product identified for sequencing (by SPA)	Ploidy level
OPA-13	RFS-135	-	-
OPA-16	K-2		
OPA-19	K-2		
OPC-8	K-2	OPC-08_{750} bp	4x
OPQ-04	S-36 & K-2	-	-
OPQ-05	K-2		
OPQ15	K-2		
OPO-12	S-36	OPO-12_{1200} bp	2x
OPO-13	K-2		
OPO-15	V-1, K-2 & S-13		
OPD-01	K-2	OPD-01_{500} bp	4x
OPD-03	S-36 & K-2		
OPD-05	S-36 & K-2		
OPD-07	S-36		
OPD-18	K-2		
OPD-20	S-36 & K-2	OPD-20_{750} bp	4x
OPS-01	V-1 & K-2		
OPS-05	K-2	OPS-05_{1100} bp	2x
OPS-11	K-2		
OPS-12	K-2		

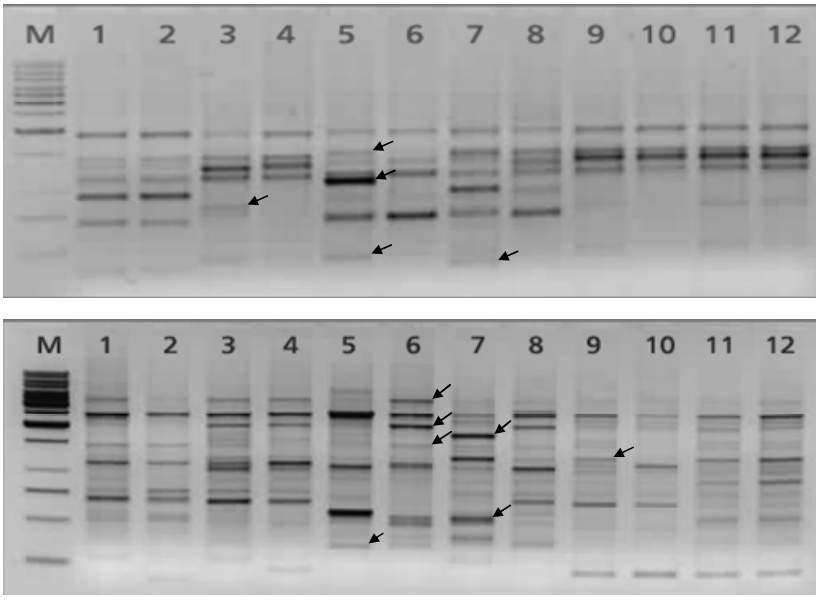

Fig. 2 Genetic profiles of 12 diploid and their colchi-tetraploid derivatives amplified by RAPD primers. (**Top gel**) OPS-11 and (**Bottom gel**) OPD-07. The lane correspond to 1) RFS-135 (2x), 2) RFS-135 (4x), 3) V-1 (2x), 4) V-1 (4x), 5) S-36 (2x), 6) S-36 (4x), 7) K-2 (2x), 8) K-2 (4x), 9) S-13 (2x), 10) S-13 (4x), 11) S-34 (2x) and 12) S-34 (4x). M is the 1 Kb molecular weight marker. The arrow indicates the marker variation observed between the diploid and its colchi-tetraploid.

Table 6 Genetic similarity indexes of mulberry genotypes.

	RFS-135-2X	RFS-135-4X	V1-2X	V1-4X	S-36-2X	S-36-4X	K2-2X	K2-4X	S-13-2X	S13-4X	S34-2X	S34-4X
RFS-135-2X	1											
RFS-135-4X	0.793103	1										
V1-2X	0.474164	0.513595	1									
V1-4X	0.498294	0.542373	0.77027	1								
S-36-2X	0.466877	0.526984	0.627219	0.550769	1							
S-36-4X	0.518382	0.532143	0.570978	0.58042	0.738182	1						
K2-2X	0.509677	0.536508	0.602899	0.508929	0.64	0.571895	1					
K2-4X	0.511551	0.590604	0.640483	0.613636	0.639498	0.57	0.638629	1				
S13-2X	0.489796	0.513333	0.703583	0.697509	0.571875	0.544521	0.542683	0.584665	1			
S13-4X	0.501845	0.532609	0.607843	0.676692	0.484277	0.521739	0.489028	0.560403	0.765873	1		
S34-2X	0.482315	0.509494	0.711599	0.666667	0.618462	0.564356	0.607903	0.626959	0.744755	0.65371	1	
S34-4X	0.481132	0.512422	0.721362	0.661238	0.619335	0.566343	0.608955	0.632716	0.731293	0.659722	0.934546	1

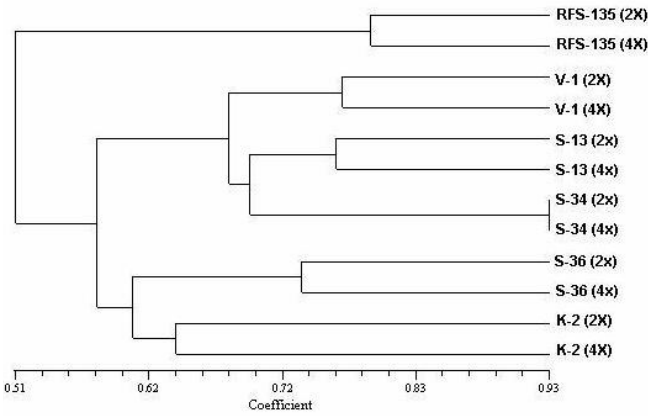

Fig. 3 UPGMA clustering of diploid and tetraploid varieties of mulberry.

The genetic profile of OPS-11 and OPD-07 is provided in **Fig. 2**. Based on the size and distinct separation pattern of the amplified products, five markers that were present in a diploid variety and absent in the colchi-tetraploid derivatives or *vice versa* were selected for sequencing by single pass analysis (**Table 5**). The size of these products as estimated on the agarose gel varied from 500 to 1200 bp.

The Dice similarity coefficients were calculated (**Table 6**) using polymorphic marker scores and were used for construction of an UPGMA dendrogram for depiction of genetic relationship among the mulberry varieties, as shown in **Fig. 3**. The clustering pattern indicated a closer relationship among the diploid and colchi-tetraploid. However, genetic variation was maximum (36%) between diploid and tetraploid of K-2 and least in case of S-34 pair (7%).

DISCUSSION

In mulberry, auto-tetraploidy can be induced by colchicine treatment. In general, the colchi-tetraploids developed do not possess economically desirable characteristics and hence cannot be directly utilized. These tetraploids show slow growth, poor rooting and often do not possess the better characteristics of the diploid variety from which they have been developed. These tetraploids possess large number of trichomes on the leaf and a thick cuticle thus making them generally unsuitable for silkworm rearing (Hamada 1963; Das *et al*. 1970; Tojyo 1985).

Tetraploids serve as a potential source for the production of triploid variants, which possess useful attributes contributing to the increased productivity in mulberry sericulture (Mustafaev 1971). The quality of silk depends on the nutritional value of the mulberry. Interference of the alkaloid colchicine with the tubulin molecules arrests the formation of spindle fibers and thus prevents the separation of chromosomes to the poles in mitosis (Blakeslee and Avery 1937). Disruption at the cell division stage reflects in variation in the nuclear content and expression of altered phenotypic characters that are also associated with the nutritional index of the plant. Induction of mutation by the alkaloid at multiple points has been observed in mulberry cultivars (Dwivedi *et al*. 1986, 1988) and other crop varieties viz., cotton (Helena 2005) and barley (Gilbert and Patterson 1965).

The comparative morphological analysis of six mulberry colchi-tetraploids and their diploid varieties exhibited variation in the parameters studied. All the parameters studied showed differences at morphological and physiological levels except for leaf apex and lobation pattern. The colchicine-treated buds showed growth of morphologically distinct leaf pattern and size in all the genotypes, which is in concordance with the early reports in mulberry ploidy breeding (Sikdar and Jolly 1994; Chakraborti *et al*. 1998). Variations in stomatal frequency were observed in all the diploid varieties. Among the diploid varieties analyzed, RFS-135 showed greater stomatal frequency, and the character in general recorded a decrease in value with an increase in chromosome number with a non-significant exception in K-2. Therefore, it is concluded that chromosome doubling in mulberry is invariably associated with a reduction in stomatal frequency (Chaicharoen *et al*. 1995). Reduction of stomatal frequency offers a greater tolerance against desiccation and a desirable attribute in evolving stress-tolerant mulberry varieties. Chloroplast count has been one of the easy methods for determining ploidy status in mulberry (Sikdar *et al*. 1986) and useful in grouping large numbers of mulberry germplasm into different ploidy groups without resorting to chromosomal preparations. The highest mean SPAD value was recorded in diploid S-34 (40.36 ± 0.37) followed by S-13 (38.2 ± 1.65), but in both cases, the character was negatively correlated with tetraploidy. From **Table 3**, it is presumed that the induction of tetraploidy is not associated with an increase in SPAD value, except in K-2 and S-36. However, most of the varieties studied (viz., RFS-135, K-2, S-36 and V-1) had an increased amount of SPAD values with an increase in the nuclear genome content. The inter-nodal distance among the varieties showed highest values in diploid variety of V-1 and its tetraploid. However, as per Pearson's correlation analysis, in general, the character is negatively correlated with an increase in ploidy level. Reduction of inter-nodal distance is a desirable trait in mulberry crop improvement as the production of leaf biomass increases per unit length of the shoot. According to DMRT all the genotypes showed significant differences in SPAD chlorophyll content and stomatal frequency except for internodal distance, which was significant among diploid varieties and insignificant in tetraploids (**Table 4**).

The present study aimed to resolve the genetic changes that are observed in mulberry phenotypes using PCR-based DNA markers. The RAPD markers used could sharply discriminate diploids from tetraploids more precisely than morphological variations. The greater discriminatory power of RAPD markers were previously exploited in mulberry

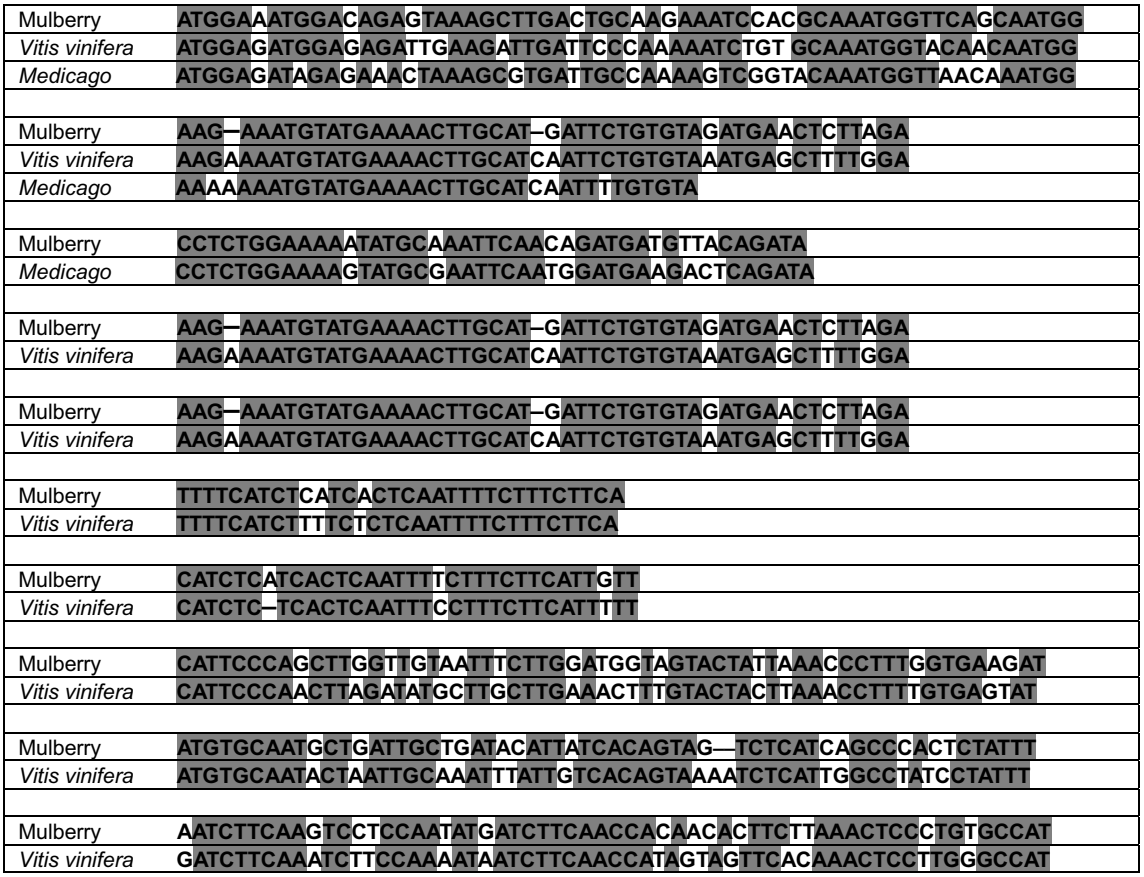

Fig. 4 Non coding sequence alignment of mulberry genome. Conserved sequences of mulberry variety K2, *Vitis vinifera* and *Medicago truncatula* are highlighted in grey.

varieties (Bhattacharya and Ranade 2001; Vijayan *et al*. 2005) and other commercial crop species like cotton (Iqbal *et al*. 1997; Rauf *et al*. 2006). A number of marker variations among the pair of diploid-tetraploid mulberry varieties were observed. It is generally assumed the colchine is mainly associated with chromosome doubling, with no other effect on the genetic structure. However, our finding on the disappearance of certain DNA markers in tetraploids when compared to the diploid progenitors provides a clue that genetic changes may take place due to colchicine treatment. This was supported by results found in rye grass (*Lolium* spp.) genotypes in which colchicine caused significant alterations in the frequency and occurrence of DNA markers (Castro *et al*. 2003). Altered phenotype of the tetraploids can also partly support the changes in the genome. A similar study in black chokeberry (*Aronia melanocarpa*) revealed results of molecular variation between diploid and tetraploids (Hovmalm *et al*. 2005). However, the genetic effect of doubling of a diploid chromosome also has a profound effect on the phenotype. Normally, one expects the *gigas* effect on doubling of normal complement of chromosomes due to high gene dosage (Das and Prasad 1973; Chaicharoen *et al.* 1995). The marker variation that was observed between a pair of diploid and tetraploid (**Fig. 1**) may be likely due to the mutation that might have occurred at the primer binding site leading to a new PCR product or the failure of amplification of the expected marker.

We have studied 5 such markers which showed variation between a pair of diploid and tetraploid by isolation, purification, and sequencing of the product to understand the possible effects of such changes. In general, from the BLAST results it is presumed that most of the changes that occurred due to the colchicine treatment possibly affected the non-coding sequence; as the marker OPC-08_{750} of K-2 (4x) showed significant alignment with *Vitis vinifera* contig VV78X139241.9, whole genome shotgun sequences and also with *Medicago truncatula* clone mth2166j21. Further, the BLAST results of the K-2 (2x) OPD-20_{750} showed significant similarities with many *Vitis vinifera*, whole genome shotgun sequences (**Fig. 4**). No significant homology with gene sequences was identified. The variation observed in non-coding sequences may be due to change in allelic frequency or presence of altered priming sites in the genome. However, it is less likely to be successful to find the genes that are associated with traits, using this approach taking into consideration the sheer size of the non-coding region of the genome.

The genetic similarity by computation of RAPD marker data indicates certain interesting clues. By and large, diploid and tetraploid pairs are very much genetically similar. However, there are certain mulberry cultivars that are very sensitive to colchicine treatment and show variation within the ploidy. This is indicated by the fact that unusually larger number marker variations are found in K-2 when compared to DNA profiles of other mulberry cultivars. This is supported by the comparatively high genetic distance between the K-2 (2x) and K-2 (4x) (**Fig. 3**). The peculiar deviation S-13 (4x) from the normal trend of having higher SPAD value and longer inter-nodal distance in case of tetraploids highlights the fact that mulberry does respond differentially to colchicine treatment. A selective mutation of specific genes controlling the trait may also be responsible for the deviation from the normal trend.

The present investigation reveals that colchicine is capable of producing variations at both the morphological and molecular levels, and the role of RAPD markers in divulging genetic variations taking place in the genome. In addition to the changes occurring in the phenotype due to chromosome doubling and gene dosage effect, there is yet another possibility of mutation affecting some of the genes governing phenotypic traits and thus adding to further variation in genetic constitution. However, further research is required for understanding the altered gene expression affecting the specific traits in auto-tetraploid mulberry.

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