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Evaluation of Genetic Diversity among Different *Melia composita* CPTs using Random Amplified Polymorphic DNA (RAPD) Markers

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Abstract

The present study was conducted to evaluate the genetic diversity among different candidate plus trees (CPTs) of *Melia composita* for providing a wider base for the further improvement of this versatile short rotation tree species. RAPD molecular markers were used to evaluate the genetic diversity in populations of *Melia composita* Willd. (Burma dek) from different agroclimatic regions. Out of the 38 decamer primers used, 13 yielded polymorphic banding patterns. In total, 105 different bands were reproducibly obtained, out of which 69 (65.7%) were polymorphic. The polymorphisms were scored and used in band-sharing analysis to identify genetic relationships. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the 24 populations into two major groups. Similarity indices ranged from 0.80 to 0.91, indicating that Burma dek germplasm within India constitutes considerably narrow genetic base. The present study reveals that PCR based fingerprinting technique, RAPD; is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different candidate plus trees (CPTs) of *Melia composita* with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. Hence the study provides a basis for tree breeders to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated with a tree crop improvement program.

Keywords: *Melia composita*, RAPD, Genetic diversity, candidate plus trees

1. Introduction

Melia composita (Willd.), commonly known as Burma dek, white cedar, Malabar neem, drek (Swaminathan et al., 2012), belongs to family Meliaceae. It is a deciduous tree, native to Indian sub-continent but now has spread in many Asian countries and also in different regions of the globe (Murugesan et al., 2013). *Melia composita* being a fast growing short rotation tree species and can be exploited for bio-energy applications especially for biomass gasification to generate producer gas and use in Lime Kiln to replace fuel oil and also for other wood product applications (Chinnaraj et al., 2011). *Melia composita*is valued for its high-quality termite and fungus resistant timber (Swaminathan et al., 2012). The multipurpose industrial wood is suitable for the manufacturing of packing cases, cigar boxes, ceiling planks, building and construction material, agricultural implements, pencils, match boxes, splints, musical instruments, tea boxes, ply board and fuel wood (Calorific value, 5.043 - 5,176 cal.) and also due its decorative appearances, which makes it suitable for furniture making (Mandang and Artistien, 2003). Every part of this versatile tree species is being used as traditional herbal medicines, such as anthelmintics, treatment of leprosy, eczema, asthma, malaria, fevers and venereal disease (Govindachari, 1992), as well as cholelithiasis, acariasis and pain (Kokwaro, 1976). Fruitsused in folk medicine as an anti-helmintic, astringent and in the treatment of colic (Kiritkar and Basu, 1999). It is well known as a rich and valuable source of bioactive limonoids (Awang et al., 2007) which are highly oxygenated and modified terpenoids having insecticidal, antibacterial, antifungal, anti-malarial, anticancer, antiviral and pharmaceutical properties (Endo et al., 2002; Koul et al., 2004).

Inspite of it's such a high value; required efforts have not been made for its genetic improvement. Genetic diversity and natural distribution pattern are very important for the introduction and conservation. The range of genetic variability is controlled by reproductive systems which in turn controls the adaptive change (Simmonds, 1962). The relative genetic diversity among individuals or populations can be determined using morphological and molecular markers. Phenotypic characters have a limited importance since they are considerably influenced by environmental factors and developmental stages of the plant and also due to the fact that

in some species adequate levels of phenotypic polymorphism are not available (Tatineni et al., 1996). On the contrary, molecular markers, based on DNA sequence polymorphism, are independent of environmental conditions and shows higher levels of polymorphism. Several methods such as isozymes or restriction fragment length polymorphisms (RFLPs) have been used in the analysis of genetic relationship in different species (Soltis and Soltis, 1990; Wang et al., 1994). Genetic diversity and natural distribution pattern are very important for the introduction and conservation.Random amplified polymorphic DNAs (RAPDs) have been widely employed for their simplicity and capacity to detect genetic variation in natural populations (Karp et al., 1998). The lack of reproducibility is considered to be its limitation, but studies show that the results of RAPD can be reproduced in a stable polymerase chain reaction (PCR) system (Wang et al., 2003). The objective of the present investigation was to evaluate genetic diversity using RAPD markers in *Melia composita.*

2. Materials and Methods

2.1. *Plant material*

Candidate plus trees (CPTs) were selected based on an intensive survey of different agroclimatic regions of Haryana, Punjab, Uttrakhand and Himachal Pradesh (Table 1). The selection was made on phenotypic assessment of desirable characters of economic interest such as stem straightness, self-pruning ability; clear bole height, low branching habit, disease resistance, etc. The juvenile (young) leaves of nursery raised progenies of different candidate plus trees formed the basic material for present study.

Table 1: Geological location of selected Plus trees of *Melia composite*

S l . No.	Asses-sion code	Geographi-cal source	Latitudes and Longitudes
1.	MCS1	Himachal Pradesh	30°50' N and 77°11'30" E
2.	MCPN1	Uttrakhand	29°N and 79.29°E
3.	MCBI1	Haryana	29°33’0” N and 75°56’0” E
4.	MCBI2	-do-	29°33’0” N and 75°56’0” E
5.	MCBI3	-do-	29°33’0” N and 75°56’0” E
6.	MCBI4	-do-	29°33’0” N and 75°56’0” E
7.	MCBI5	-do-	29°33’0” N and 75°56’0” E
8.	MCP1	Punjab	30°65’N, and 75°69’ E
9.	MCP3	-do-	24°20' N and 72° 44 E
10.	MCP5	-do-	24°20' N and 72° 44 E
11.	MCP6	-do-	21°39’N and 73°43 E
12.	MCP7	-do-	21°39’N and 73°43 E
13.	MCP10	-do-	30°42'N and 75°18' E
14.	MCP11	-do-	30°42'N and 75°18' E
15.	MCP12	-do-	30°42'N and 75°18' E
16.	MCP14	-do-	29°43'N and 76°13' E
17.	MCP15	-do-	29°43'N and 76°13' E
18.	MCPAU1	Punjab	30°90’N and 75°81’E
19.	MCPAU2	-do-	30°90’N and 75°81’E
20.	MCPAU3	-do-	30°90’N and 75°81’E
21.	MCPAU5	-do-	30°90’N and 75°81’E
22.	MCK1	Haryana	29.96° N, 76.83° E
23.	MCK2	-do-	29.96° N, 76.83° E
24.	MCB1	Punjab	30° 10’ N and 75°09’ E

2.2. *DNA extraction*

DNA was isolated following the protocol of Murray and Thompson (1980), modified by Saghai-Maroof et al. (1984) and Xu et al. (1994). Fresh young leaves from individual nursery grown progenies of plus trees were collected in icebox. Three to four grams of tissue was ground to a very fine powder in liquid nitrogen using mortar and pestle. The powder was transferred to a 50 ml polypropylene centrifuge tube containing 20 ml of pre-warmed (60 °C) DNA extraction buffer (3.5 g CTAB; 5 M NaCl; 0.5 M EDTA, pH 8.0; 1 M Tris, pH 8.0; 2 ml β-mercaptoethanol). The suspension was incubated in water bath at 65 °C for 2 hours with intermittent swirling. After incubation, the mixture was emulsified with an equal volume of chloroform: iso-amyl alcohol mixture (24:1) for 10-15 minutes by swirling. The content was centrifuged at 10,000 rpm for 20 minutes at room temperature. The upper aqueous phase (supernatant) was transferred in a pre-sterilized centrifuged tube and again extracted with an equal volume of chloroform: iso-amyl alcohol (24:1). Thus, following centrifugation, the aqueous phase was collected and mixed with equal volume of chilled isopropanol and was left for over-night at -20 °C temperature. The precipitated DNA was spooled out with help of micro-pipette and was centrifuged at 13,000 rpm for 15 minutes at 4 °C temperature to form DNA pellets. After pellet formation, DNA was washed with help of 70% alcohol for 2 times. The washed DNA was dried at room temperature for 4-6 hrs and was subsequently dissolved in 100 µl of TE (Tris 10mM, EDTA 1mM) buffer.

DNA Quantification

Quantification of DNA was accomplished by analyzing the purified DNA on 1% agarose gel alongside diluter uncut lambda DNA as standard. DNA was diluted in TE to a concentration ofapproximately 12.5 ng/µl for use in PCR analysis.

2.3. *DNA amplification*

A total of 38 RAPD primers from Life Technologies Corporation (Table 2) were used for PCR amplification.PCR amplification were performed using 96 well thermal cycler (Quanta biotech, Model S20143) using 20 µl PCR reaction mixture containing 1 X PCR buffer (50 mMKCl, 10 mMTris-Cl, 1.5 mM $MgCl_2$), 200

Table 2: Lists of RAPD primers and their annealing and melting temperatures used in the present investigation

Sl. No.	Primer	Sequence (5'-3')	G+C content (%)	Melting temperature (Tm)	Annealing temperature (Ta)
1.	MA 16	TGAGCCTCAC	55	33.7	37.3
2.	MA 18	GGTGCGGGAA	55	40.4	40.8
3.	MA 19	CCAGATGCAC	70	32.6	35.7
4.	MA 20	GTGACATGCC	60	33.2	33.4
5.	MA 21	TCAGGGAGGT	65	33.9	34.9
6.	MA 24	CACCAGGTGA	55	33.4	38.3
7.	MA 25	GAGTCTCAGG	75	29.5	31.9
8.	MA 26	GGTGACTGTG	55	31.8	35.7
9.	MA 27	GGCACGTAAG	60	32.9	37.7
10.	MA 28	AAGTCCGCTC	60	34.6	34.1
11.	MA 29	CCCAGTCACT	65	32.8	37.3
12.	MA 30	CCACGGGAAG	55	36.6	40.0
13.	MA 31	CAGCACTGAC	70	32.7	41.5
14.	MA 32	TCAGAGCGCC	65	40.1	35.4
15.	MA 33	GACAGGAGGT	65	32.2	30.3
16.	MA 34	GTCCACTGTG	60	31.8	32.2
17.	MA 35	GAGGATCCCT	70	30.9	32.2
18.	MA 36	GTCGCCGTCA	70	40.8	41.4
19.	MA 37	TCTGGTGAGG	60	32.2	37.9
20.	MA 38	CACCGTATCC	60	38.0	33.5
21.	MA 39	CATCCGTGCT	65	35.8	40.1
22.	MA 40	AGGGCGTAAG	55	33.9	38.3
23.	MA 41	CCAAGCTTCC	67	32.3	34.1
24.	MA 42	TGCCCGTCGT	55	43.2	45.1
25.	MA 43	GGAAGCTTGG	65	32.3	33.3
26.	MA 44	GGGACGATGG	60	36.2	31.6
27.	MA 45	AGCAGGTGGA	56	36.0	37.9
28.	MA 46	TGTCATCCCC	55	32.8	34.3
29.	LD 3230	AATCGGGCTG	63	32.0	33.5
30.	LD 3231	CAATCGCCGT	58	32.0	35.1
31.	LD 3233	GACCGCTTGT	75	32.0	31.1
32.	LD 3235	TCGCCGCAAA	70	32.0	30.7
33.	LD 3236	AGCGTCACTC	60	32.0	36.6
34.	LD 3247	AAAGCTGCGG	65	32.0	36.5
35.	LD 3248	GACGGATCAG	70	32.0	30.5
36.	LD 3252	GTTGCCACCC	62	34.0	38.3
37.	LD 3256	GTCCACACGG	65	34.0	37.2
38.	LD 3258	CTGCTGGGAC	60	34.0	36.1

µM each of dNTPs, 20 ng of primer, 0.45 unit of Taq DNA polymerase (New England Biolabs, Pvt., Ltd.) and 50 ng of DNA template. The first cycle consisted of denaturation of template DNA at 94 °C for 4 min, primer annealing (37 °C) for

1 min and primer extension (72 °C) for 1 min. in the next 47 cycles, the period of denaturation was reduced to 1 min while the primer annealing and the primer extension time remained as in the first cycle. The last cycle consisted of only primer extension (72 °C) for 10 min. Amplified DNA fragments were separated on a 1.5% agarose gel in 1X Tris-Borate EDTA buffer. The size (bp) of most intensely amplified band for each RAPD marker was determined based on its migration relative to molecular weight of DNA ladder (Gene Ruler ™ 1Kb and 10bp DNA Ladder). The DNA bands were visualized by staining the gels with nucleic acid stain and photographed under UV light using gel documentation system (Alpha Innotech Corporation, California, USA). The experiments were repeated at least twice to confirm the results.

2.4. *Band scoring and data analysis*

The frequency of RAPD polymorphism between 24 genotypes of Burma dek was calculated based on the presence of band '1' or absence of band '0'. The genetic association among genotypes was evaluated by calculating the Jaccard's similarity co-efficient for pair-wise comparison based on the proportions of shared bands produced by primers. Similarity matrix was generated using 'Simqual' sub-program of software NTSYS-PC (Rohlf, 1990). Jaccard similarity coefficients were used for cluster analysis of genotypes using 'SAHN' sub-program and dendrogram based on UPGMA. Principle component analysis (NTSYS-PC) was performed to generate a two and three dimensional representation of genetic relationship among 24 genotypes of *Melia composita*.

3. Results and Discussion

Out of 38 decamer primers screened, only 13 were found to produce intensely stained and reproducible polymorphic bands among 24 genotypes of *Melia composita* while the rest of the primers resulted in either no amplification or smeared profiles. A total 105 amplified bands were obtained, out of which 36 were monomorphic and 69 were polymorphic (Table 3). Average polymorphism across all the 24 genotypes of *M.composita* was found to be 65.71%. The size of amplified products ranged from 200-4937 bp in size. Such a high level of polymorphism might be due to the use of primers with high GC content. The GC content is an important criterion for the selection of primers because it is associated with Tm (annealing temperature) and related to the reproducibility of results (Dhillon et al., 2012). The higher GC contents produced higher frequencies of RAPD because of increase in total frequencies of amplified fragments. The number of bands (fragments) per primer ranged from 4 (MA 33) to 11 (MA 39, MA 45), the average number of bands per primer being 8.

The pair-wise comparison of the RAPD profiles based on both shared and unique amplification products were made

Table 3: Random primers showing polymorphism among *Melia composita* genotypes

Primer code	Nucleotide sequence (5'-3')	Total No. of amplified fragments	No. of monomorphic fragments	No. of polymorphic fragments	Fragment size range (bp)	PIC values
MA 16	TGAGCCTCAC	7	3	4	425 – 3247	0.67
MA 28	AAGTCCGCTC	8	5	3	252 – 4509	0.87
MA 29	CCCAGTCACT	6	2	4	418 – 4454	0.64
MA 30	CCACGGGAAG	9	4	5	255 – 4937	0.55
MA 31	CAGCACTGAC	7	4	3	200 – 3856	0.60
MA 32	TCAGAGCGCC	10	4	6	450 – 4869	0.41
MA 33	GACAGGAGGT	4	1	3	327 – 4416	0.63
MA 36	GTCGCCGTCA	8	3	5	395 – 3982	0.35
MA 39	CATCCGTGCT	11	1	10	350 – 3407	0.57
MA 41	CCAAGCTTCC	8	3	5	404 – 3916	0.82
MA 44	GGGACGATGG	8	3	5	350 – 3676	0.62
MA 45	AGCAGGTGGA	11	3	8	433 – 4201	0.52
LD 3256	GTCCACACGG	8	0	8	307 – 2992	0.75
Total		105	36	69		

to generate a similarity matrix. The mean genetic similarity as obtained from similarity matrix (Table 4) varied from 80.3 to of 91.2% within 24 genotypes. The maximum similarity value (0.91) was observed between MCPN1 and MCPAU3 genotypes selected from Uttrakhand and Punjab, respectively indicating these genotypes to be most closely related genotypes. The climatic conditions of these places are drastically different but these conditions don't seem to have played a role in evolving genetic diversity in *Melia composita*. However, the lower value of the similarity coefficient was observed between MCBI4 and MCP10 (80.3) genotypes selected from Haryana and Punjab, respectively. Such a narrow range in similarity co-efficient

Table 4: Simple matching coefficients of similarity among 24 genotypes of *Melia composita*

	MCP3	MCP5	MCP6	MCP7	MCP10	MCP11	MCP12	MCP14	MCP15	MCS1	MCPAU1	MCPAU2
MCP3	1											
MCP5	0.87	1										
MCP6	0.86	0.87	1									
MCP7	0.84	0.86	0.85	1								
MCP10	0.81	0.85	0.82	0.85	1							
MCP11	0.82	0.85	0.83	0.84	0.84	1						
MCP12	0.84	0.87	0.84	0.87	0.84	0.86	1					
MCP14	0.83	0.86	0.85	0.85	0.84	0.84	0.87	1				
MCP15	0.83	0.85	0.85	0.84	0.83	0.83	0.85	0.85	1			
MCS1	0.83	0.85	0.84	0.84	0.84	0.84	0.86	0.84	0.86	1		
MCPAU1	0.86	0.88	0.86	0.86	0.84	0.85	0.88	0.86	0.87	0.88	1	
MCPAU2	0.87	0.87	0.86	0.85	0.84	0.84	0.85	0.85	0.85	0.87	0.91	1
MCPAU5	0.86	0.88	0.86	0.86	0.85	0.85	0.86	0.86	0.86	0.88	0.90	0.89
MCB1	0.84	0.85	0.84	0.85	0.83	0.83	0.84	0.84	0.84	0.85	0.87	0.86
MCPN1	0.85	0.87	0.86	0.85	0.84	0.85	0.86	0.86	0.85	0.86	0.89	0.88
MCPAU3	0.85	0.86	0.85	0.85	0.84	0.84	0.85	0.85	0.84	0.85	0.88	0.87
MCBI1	0.85	0.87	0.85	0.85	0.83	0.84	0.85	0.85	0.85	0.86	0.88	0.88
MCBI2	0.82	0.83	0.81	0.83	0.80	0.81	0.82	0.81	0.81	0.83	0.85	0.84
MCBI3	0.83	0.86	0.84	0.84	0.82	0.83	0.85	0.84	0.84	0.85	0.87	0.86
MCBI4	0.81	0.84	0.82	0.82	0.8	0.81	0.83	0.82	0.81	0.83	0.85	0.84
MCBI5	0.82	0.84	0.83	0.83	0.81	0.82	0.84	0.83	0.83	0.84	0.86	0.85
MCK1	0.85	0.87	0.86	0.86	0.84	0.85	0.87	0.86	0.86	0.88	0.89	0.88
MCK2	0.83	0.85	0.83	0.83	0.81	0.82	0.84	0.83	0.83	0.84	0.87	0.86
MCP1	0.84	0.86	0.85	0.84	0.82	0.83	0.84	0.85	0.84	0.85	0.87	0.87

Table 4: Continue...

	MCPAU5	MCB1	MCPN1	MCPAU3	MCBI1	MCBI2	MCBI3	MCBI4	MCBI5	MCK1	MCK2	MCP1
MCPAU5	1											
MCB1	0.88	1										
MCPN1	0.89	0.89	1									
MCPAU3	0.88	0.89	0.91	1								
MCBI1	0.89	0.89	0.90	0.89	1							
MCBI2	0.85	0.85	0.87	0.88	0.87	1						
MCBI3	0.87	0.88	0.90	0.90	0.89	0.87	1					
MCBI4	0.85	0.85	0.88	0.88	0.87	0.87	0.90	1				
MCBI5	0.86	0.85	0.88	0.87	0.87	0.84	0.88	0.86	1			
MCK1	0.89	0.88	0.91	0.90	0.91	0.86	0.90	0.88	0.90	1		
MCK2	0.87	0.86	0.88	0.87	0.87	0.85	0.87	0.86	0.86	0.89	1	
MCP1	0.88	0.87	0.90	0.88	0.89	0.86	0.89	0.88	0.88	0.90	0.89	1

values suggests that the Burma dek germplasm collection represents a genetically identical population. One of the major contributory factors to the narrow range of similarity co-efficient observed in Burma dek may be on account of

its evolutionary status as apredominantly self-pollinated species. Johar et al. (2015) has reported the predominantly self pollinated behaviour while studying the phenological and reproductive behaviour of *Melia composita*. Similar finding have already been reported by Dhillon et al. (2012) in *Jatropha curcas.*

The cluster tree analysis (dendrogram) (Figure 1) in present investigation showed the broad division of 24 genotypes into 2 major clusters between similarity coefficients of 0.84 to 0.92. Cluster I comprised of two out grouped accessions

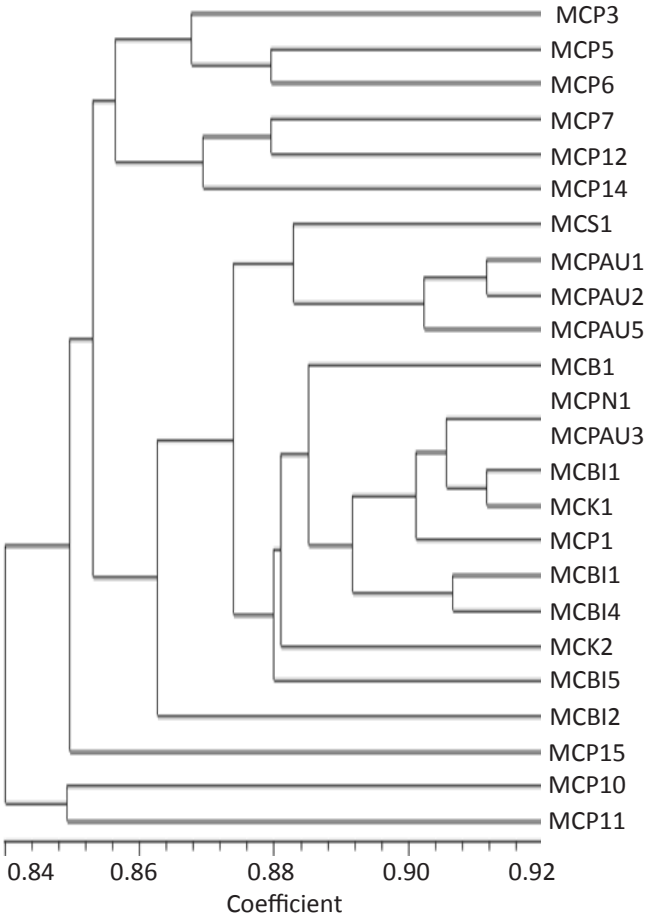

Figure 1: Dendrogram showing genetic relationship among 24 genotypesof Melia composita based on 38 RAPD primers

(MCP10 and MCP11) and cluster II consisted of the rest of 22 accessions/genotypes. Clustering together of accessions from different regions indicates lack of gene flow between adjacent populations in each region. This assumption has seen further supported by Farooqui et al. (1998). Relationship among accession/genotypes was also resolved by Principal Component Analysis (PCA). The overall grouping pattern of PCA corresponded well with the clustering pattern of dendrogram. Accession MCP10 and MCP11 were out grouped in the dendrogram, were occupying the periphery position in 2-D and 3-D PCA (Figure 2 and Figure 3). Rest of the genotypes were grouped into one main group. Hence, PCA showed a further

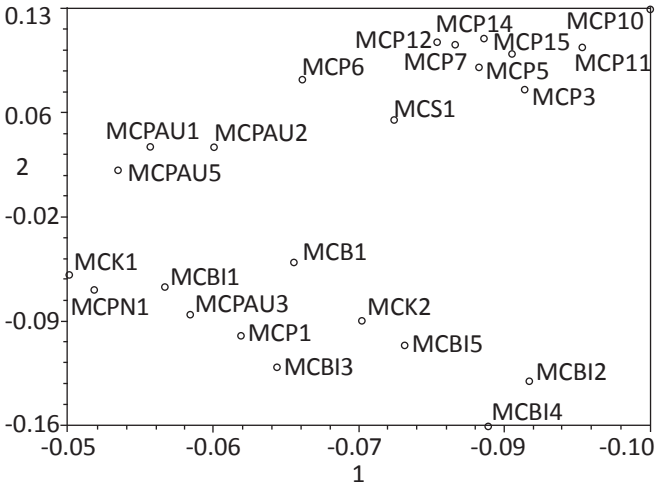

Figure 2: Two dimensional PCA scaling of 24 genotypes of Melia composita using 38 RAPD primers

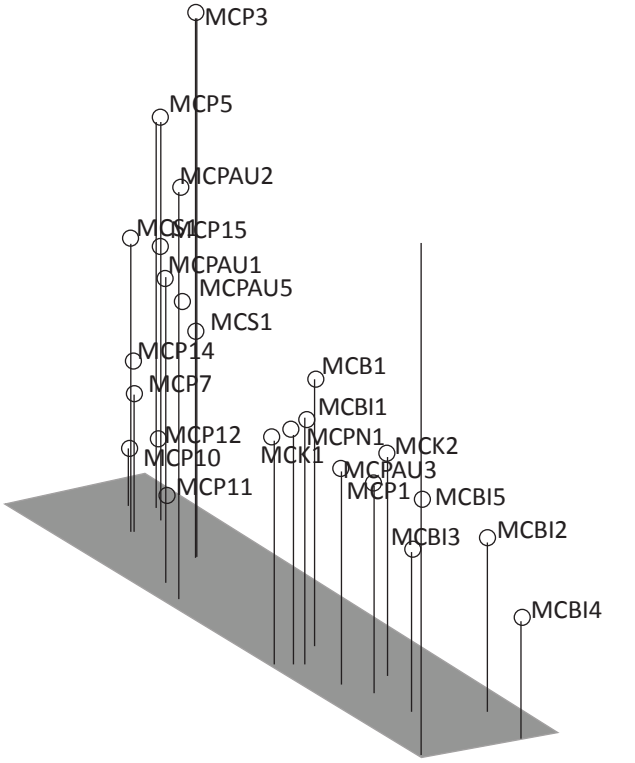

Figure 3: Three dimensional PCA scaling of 24 genotypes of Melia composita using 38 RAPD primers

confirmation of genetic similarities delineated employing UPGMA. Cluster analysis of genotypes on *Melia composita* employing UPGMA as well as principal component analysis led to the segregation of the accessions into the distinct groups, which reflected their geographical distribution. Variation in genetic diversity within species is usually related with geographical range, mode of reproduction, mating system, seed dispersal and fecundity (Loveless, 1992). The level of genetic variation detected within the Burma dek accessions with RAPD analysis suggested that it is an efficient marker

technology for delineating genetic relationships among genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategies for management, conservation and tree improvement programme.

4. Conclusion

PCR based fingerprinting technique, RAPD; is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different candidate plus trees (CPTs) of *Melia composita* with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The genetic similarity among different Candidate plus trees is high as indicated by RAPD analysis which shows a narrow genetic base. Hence the study provides a basis for tree breeders to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated with a tree crop improvement program.

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