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Research Article

Analysis of Genetic Diversity of Neem Using RAPD Markers

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ABSTRACT

The present investigation was carried out during 2013-14. RAPD molecular markers were used to evaluate the genetic diversity in populations of Azadirachta indica A. Juss (neem) from different regions of north Karnataka. Out of the 05 random decamer primers used, 03 yielded polymorphic banding patterns. In total, 23 different DNA bands were reproducibly obtained, out of which 18 (78.26%) were polymorphic. The polymorphisms were scored and data was plotted as a binary matrix to be used for phylogenetic analysis using PyElph 1.4 and PAST software. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the 20 populations into two major groups. The principal component analysis observed on an average 37% of the total variation.

Keyword: *Azadirachta indica, genetic variation, DNA, RAPD, PCR.*

INTRODUCTION

Various types of molecular markers are utilized to evaluate DNA polymorphism. RAPD a PCR based technique is commonly used by plant biologists to perform a number of tasks, including the genetic fingerprinting of plant varieties, determining similarities among inbred varieties, mapping of plant genomes, and establishing phylogeny among plant species. Although, RAPD is criticized for its low reproducibility (Hansen et al., 1998 & Virk et al., 2000), it is overcome by optimization of the RAPD reaction and conditions. Large scale characterization of plant species in varying geoclimatic conditions can be performed using various parameters such as seed morphometric traits and isozymes (Kundu, 1999). However, environmental factors as well as the developmental stages of the plants influence such traits. DNA based markers provide new tool for ecological and genetic studies of evolutionary processes (Cruzan, 1998).

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RAPD markers have successfully been used to estimate levels of relatedness among the individuals, studies of mating systems, and seed dispersal and seedling establishment in natural populations.

The objective of this study is to evaluate the genetic variations and genetic relationships among the neem accessions collected from four ecological regions on the basis of RAPD.

MATERIALS AND METHODS

Plant material

Twenty seed samples of *Azadirachta indica* were collected from four different regions, namely Bidar, Gulbarga, Raichur from Hyderabad-Karnataka (HK) region and Zaheerabad, Telangana. All the populations were studied for the genetic variation and genetic relationship by RAPD.

Isolation of genomic DNA

200mg of Seeds were ground to powder with liquid nitrogen using a mortar and pestle. It was suspended in CTAB (Cethyltrimethyl ammonium bromide) extraction buffer modified method of Rahmatollah et al. 2014. The extraction buffer contained (2% CTAB, 5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl, pH 8.0, and 0.1% 2-mercaptoethanol, 0.1% activated charcoal). The sample was transferred to the Eppendorf tube and vertexes at 5-10 times. Then tubes were centrifuged at 10,000 rpm for 15 min. Supernatant was transferred to the new Eppendorf tubes and 800 µl of chloroform: isoamylalcohol (24:1) was added. The content were mixed gently by vertexing and centrifuged at 10,000 rpm for 15 min. The aqueous layer was collected and precipitated by adding 0.8 volumes of cold isopropanol. Mixed by inverting subsequently, the tubes were incubated at -20^{0}C for 1 hour. The DNA was pelleted by centrifugation at 10,000 rpm for 15 min. The supernatants were removed and the DNA pellet washed with 700 µl of 70% ethanol, repeating this wash for two times. The pellet was dried for 10 minutes and then dissolved in 200 µl of TE buffer. After DNA extraction, the existence and concentration of DNA were checked by agarose gel electrophoresis.

Purification of Genomic DNA by RNase and Proteinase treatment

The isolated genomic DNA was further purified by treating with RNase to remove RNA. To 100 µl of isolated DNA sample, 5 µl of RNase (5 mg/ml) was added and incubated at 37^{0}C for 1 h. After incubation with RNase, Proteinase K treatment was done to make the DNA free from proteins. To the RNase treated 100 µl DNA, 0.25 µl of Proteinase K (20 mg/ml) was added and incubated at 37^{0}C for 30 min. The reaction mixture was made up to 500 µl with TE buffer and extracted with saturated phenol, chloroform, isoamyl alcohol treatment.

Analysis of RAPD

RAPD-PCR analysis of isolated DNA was performed by five random decamer primes, which were procured from Eurofins Genomics India Pvt Ltd., Bengaluru. The primers and their sequences are listed in Table 1. The molecular biology chemicals required for RAPD-PCR analysis are Taq DNA polymerase, Taq DNA assay buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), dNTPs (dATP, dCTP, dGTP and dTTP) were purchased from Bangalore Genei Pvt. Ltd., Bangalore. The DNA isolated from 20 neem ecotypes were diluted to a concentration of 20 ng/µl, RAPD-PCR amplifications were performed in 20 µl reaction mixture, as described by Dhillon et al. (2007). The reaction mixture contained following components in 0.5 ml PCR tube (Table 2). The reaction mixture was thoroughly mixed and centrifuged the samples briefly to bring down the contents of the tube. To this 50 µl of mineral oil was overlaid to prevent the evaporation of the contents during the amplification process. The DNA thermal cycler Corbett was used with following standardized program (Table 3).

The RAPD-PCR amplified products were visualized by ethidium bromide and banding pattern were stored for further analysis under gel documentation system and photographs were taken.

Data Analysis

For data analysis of RAPD-PCR, only distinct visible and reproducible bands were manually scored, '1' for presence and '0' for the absence and the binary data were used for statistical analysis by using the software "PAST". The sizes of the fragments (molecular weight in base pairs) were estimated by using 100 bp ladder markers, which was run along with the amplified products. A genetic dissimilarity matrix was calculated according to Jaccard's similarity index (JSI) which estimated all pairwise differences in the amplification product and cluster analysis was done by the UPGMA method using the variance algorithm in PhyElp 1.4 software.

Table 1: List of RAPD primers used for the amplification of genomic DNA of neem seed

Primer code	Nucleotide sequence (5'→3')
OPA-5	AGGGGTCTTG
OPC-8	TGGACCGGTG
OPA-19	CAAACGTCGG
OPA-03	AGTCAGCCAC
OPQ-17	GAAGCCCTTG

Table 2: Reaction mixture of PCR amplification

Constituents	Quantity (µl)	Final concentration
DNA	2.0	25-30ng
Taq DNA assay buffer	2.0	1X
dNTPs	0.4	200 µM each dNTPs
Primer	2.0	20 picomoles
Taq DNA polymerase	0.3	1 Unit
1.5 M $MgCl_2$	1.0	15 mM
Sterile water	12.3	20 µl

Table 3: PCR amplification cycles

Process	Temperature (^{0}C)	Time (min)	No. of cycles
Initial denaturation	94	4	1
Denaturation	94	1	45
Annealing	37	1	
Extension	72	2	
Final extension	72	10	1
Storage	4	Forever	Forever

RESULTS

Isolation of genomic DNA from neem seeds

The isolated DNA was subjected to check their purity at A_{260}/A_{280} which were found 1.91 that indicated the isolated DNA was contaminated with RNA which were further purified by RNase treatment. The DNA yielded from the neem was 8-10 µg/g of seed material. The electrophoresis pattern of isolated genomic DNA after RNase treatment is shown in the Fig. 1.

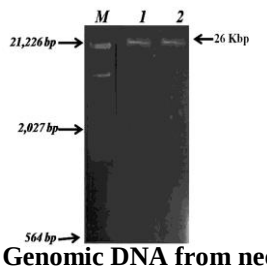

Fig. 1: Genomic DNA from neem seeds

Genetic diversity analysis by RAPD Marker

In the present study 20 neem ecotypes (Table 4) were subjected for RAPD analysis with 5 random decamer primers, out of which 3 primers were produced considerable polymorphism while 2 primers were either not able to produce the bands or had weaker amplification. The high intensity and reproducible bands were only selected for polymorphism analysis among the ecotypes. Differing band intensities were not taken into account to avoid errors.

Five primers used OPA-5, OPC-8, OPA-19, OPA-03 and OPQ-17. No bands were generated in the primer OPC-8 and OPA-19. Primer OPA-5 gives 8 different bands, in which 6 are polymorphic band. Primer OPA-03 gives 7 different bands, in which 5 are polymorphic band. Primer OPQ-17 gives 8 different bands. Out of these 7 bands are polymorphic band. The 5 primers generated different bands with size ranging from 200-2000 bp. No PCR product was generated through the primer OPC-8 and OPA-19, indicating that no binding sites for this primer. Out of the 23 bands, 18 Scorable bands (78.26%) were found to be polymorphic. These results gave an average of polymorphic bands per primer was shown in Table 5. The gel images were analyzed for the presence and absence of bands and this data was plotted as a binary matrix to be used for phylogenetic analysis using PyElph 1.4 and PAST software. PyElph 1.4 and PAST (PAleontological STatistics) free software tool, runs on standard Windows computers.

Table 4: Neem ecotypes used for RAPD analysis

Lane No.	Accessions	Ecotype region	Lane No.	Accessions	Ecotype region
1	B1	Bidar	11	R1	Raichur
2	B2		12	R2	
3	B3		13	R3	
4	B4		14	R4	
5	B5		15	R5	
6	G1	Gulbarga	16	Z1	Zaheerabad
7	G2		17	Z2	
8	G3		18	Z3	
9	G4		19	Z4	
10	G5		20	Z5	

Table 5: RAPD primers and level of polymorphism

Sl.No.	Primer code	Total no. of bands scored	No. of polymorphic bands	Proportion of polymorphism
1	OPA-5	8	6	75%
2	OPA-03	7	5	71.42%
3	OPQ-17	8	7	87.5%
	Total	**23**	**18**	**78.26%**
	Average	**7.6**	**6**	**78.94%**

Diversity analysis by OPA-5 primer (Fig. 2)

The scored data were used for calculating similarity indices by using the PAST software to generate pairwise matrix. The pairwise matrix of genetic distances was then employed to draw the dendrogram produced by Unweighted Pair Group Method with Arithmetic Averages (UPGMA) by the PhyElp 1.4 software, in order to study the precise relationships between the neem genotypes and also for cluster analysis, for grouping the neem ecotypes. The phylogenetic tree generated by PhyElp 1.4 using the UPGMA algorithm, were clustered into two major clusters - clusters 'I' and 'II'. Major cluster 'I' was divided into two sub-clusters 'Ia' and 'Ib'. Sub-cluster 'Ia' further divided into 'Ic' and 'Id'. Major cluster 'II' was bifolious comprising of Z1 and B5

accession (Fig. 3). The sub cluster 'Ic' contained six genotypes *viz*. R1, G3, G4, R4, G5 and R5. Subcluster 'Id' was the simplicifolious containing Z3. Cluster 'Ib' comprised of two sub cluster 'Ie' and 'If'. Sub cluster 'Ie' is again sub divided in to two sub clusters 'Ig' and 'Ih'. The sub cluster 'Ig' is further divided into two clusters containing five genotypes viz. Z4, R3, Z5, R2 and G2 while subcluster 'Ih' gave two clusters containing four genotypes viz. B3, B1, Z2 and B2. Subcluster 'If' comprised of staminate lines G1 and B4. The Jaccard's similarity analysis, among 20 neem ecotypes exhibited genetic similarity range of 0.0 to 1.0. Maximum similarity was observed to be 1.0 between B3-B1, G1-B5, G4-B2, R5-B1/B3 and Z5-Z4 while B5-B2, G1-B2, G2-B5, G3-B1/B3, G4-B5/G1, G5-G3 and R5-G3 gave 0.83, 0.83, 0.83, 0.87, 0.83, 0.87 and 0.87 similarities respectively (Table 6). The lowest genetic similarity noted was 0.0 in G3-G4/R1/G1 accession. In the principal component analysis all the analyzed samples were separated, which is coherent with phenogram generated by UPGMA cluster analysis. The principal component explained 40.22 percent of total variation (Fig. 4).

Diversity analysis by OPA-03 primer (Fig. 5)

The phylogenetic tree generated by PhyElp 1.4 using the UPGMA algorithm, were clustered into two major clusters - clusters 'I' and 'II' (Fig. 6). Major cluster 'I' was divided into two sub-clusters 'Ia' and 'Ib'. Sub-cluster 'Ia' further divided into 'Ic' and 'Id'. 'Ic' is further bifurcated into two groups 'Ie' and 'If'. Major cluster 'II' was the simplicifolious containing Z1. The sub cluster 'Ie' contained eight genotypes *viz*. R1, G4, R4, R2, Z2, G3, R3 and G5. Subcluster 'If' is further divided into two clusters, comprised of six genotypes viz. B4, B1, B2, G1, B3 and B5. Cluster 'Id' containing two clusters and comprised of three genotypes namely Z3, G2 and R5. Sub cluster 'Ib' comprised of staminate lines Z5 and Z4.

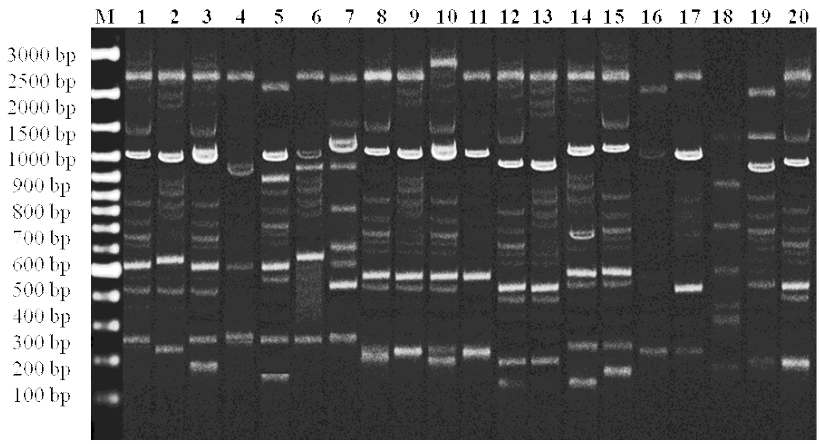

Fig. 2: RAPD banding pattern by OPA-5 primer
Lane M: GeneRuler™ 100 bp plus ladder; Lane 1-20: Neem ecotypes

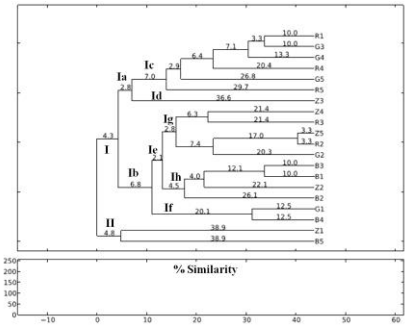

Fig 3: Phylogenetic tree generated using PhyElp 1.4 of Primer OPA-5

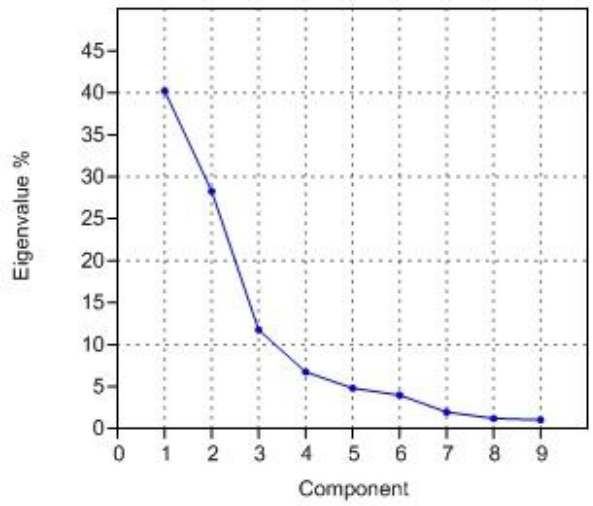

Fig. 4: Principal component analysis for primer OPA-5

Table 6: Jaccard's Similarity Matrix of gel banding pattern of Primer: OPA-5

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	**1**																			
2	0.71	**1**																		
3	**1**	0.71	**1**																	
4	0.25	0.33	0.25	**1**																
5	0.62	0.83	0.62	0.5	**1**															
6	0.62	0.83	0.62	0.5	**1**	**1**														
7	0.5	0.66	0.5	0.6	0.83	0.83	**1**													
8	0.87	0.62	0.87	0.22	0.55	0.55	0.44	**1**												
9	0.71	**1**	0.71	0.33	0.83	0.83	0.66	0.62	**1**											
10	0.75	0.5	0.75	0.11	0.44	0.44	0.33	0.87	0.5	**1**										
11	0.57	0.8	0.57	0.4	0.66	0.66	0.8	0.5	0.8	0.37	**1**									
12	0.25	0.14	0.25	0.5	0.28	0.28	0.33	0.22	0.14	0.11	0.16	**1**								
13	0.25	0.33	0.25	0.5	0.5	0.5	0.33	0.22	0.33	0.11	0.16	0.5	**1**							
14	0.71	0.66	0.71	0.33	0.57	0.57	0.66	0.62	0.66	0.5	0.8	0.14	0.14	**1**						
15	**1**	0.71	**1**	0.25	0.62	0.62	0.5	0.87	0.71	0.75	0.57	0.25	0.25	0.71	**1**					
16	0.28	0.4	0.28	0.66	0.33	0.33	0.4	0.25	0.4	0.12	0.5	0.25	0.25	0.4	0.28	**1**				
17	0.42	0.6	0.42	0.5	0.5	0.5	0.6	0.37	0.6	0.25	0.75	0.2	0.2	0.6	0.42	0.66	**1**			
18	0.12	**0**	0.12	0.25	0.14	0.14	0.16	0.11	**0**	0.12	**0**	0.25	0.25	0.16	0.12	**0**	**0**	**1**		
19	0.5	0.25	0.5	0.33	0.37	0.37	0.25	0.44	0.25	0.33	0.12	0.6	0.6	0.25	0.5	0.16	0.14	0.4	**1**	
20	0.5	0.25	0.5	0.33	0.37	0.37	0.25	0.44	0.25	0.33	0.125	0.6	0.6	0.25	0.5	0.16	0.14	0.4	**1**	**1**

The Jaccard's similarity analysis, among 20 neem ecotypes exhibited genetic similarity range of 0.0 to 1.0. Maximum similarity was observed to be 1.0 between B2-B1, G1-B3 and R5-R3 while R4-R3, R5-R4, B4-B1, B4-B2, R3-R1, R5-R1, Z2-G3, Z4-G2 and Z5-R4 gave 0.85, 0.85, 0.75, 0.75, 0.75, 0.75, 0.75, 0.71 and 0.71 similarities respectively (Table 7). The lowest genetic similarity noted was 0.0 in Z3-B1/B2/B4/B5 /G3/G4/G5/R2/Z1/Z2, Z4-B1 and Z4-B2. In the principal component analysis, all the analyzed samples were separated, which is coherent with phenogram generated by UPGMA cluster analysis. The principal component explained 30.66 percent of total variation (Fig. 7).

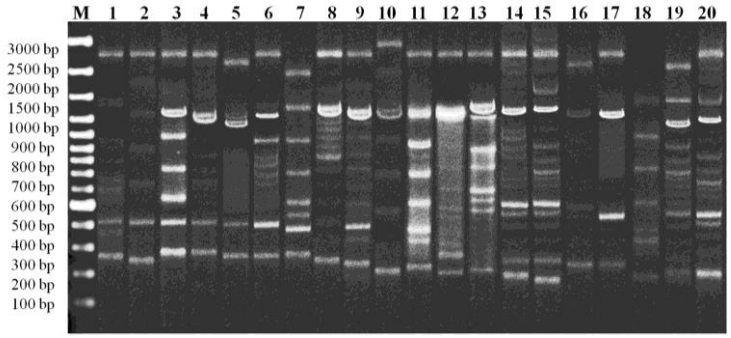

Fig. 5: RAPD banding pattern by OPA-03 primer
Lane M: GeneRuler™ 100 bp plus ladder; Lane 1-20: Neem ecotypes

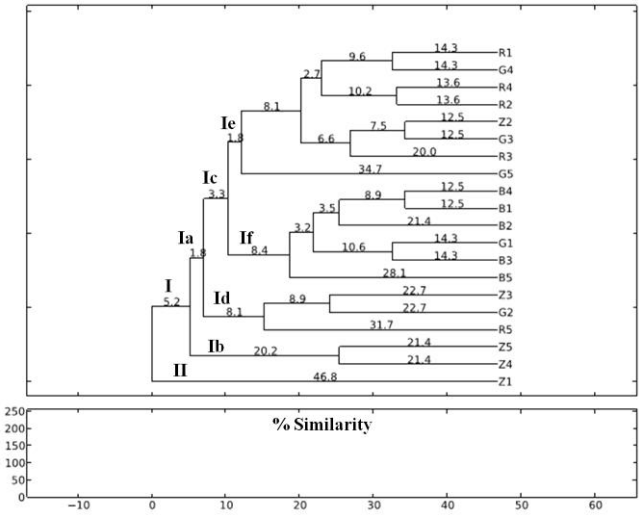

Fig. 6: Phylogenetic tree generated using PhyElp 1.4 of Primer OPA-03

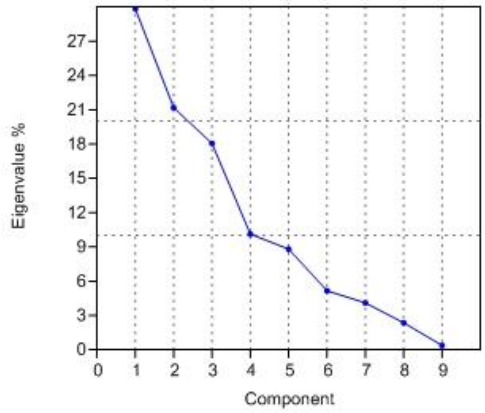

Fig. 7: Principal component analysis for primer OPA-03

Table 7: Jaccard's Similarity Matrix of gel banding pattern of Primer: OPA-03

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	**1**																			
2	**1**	**1**																		
3	0.5	0.5	**1**																	
4	0.75	0.75	0.66	**1**																
5	0.4	0.4	0.42	0.6	**1**															
6	0.5	0.5	**1**	0.66	0.42	**1**														
7	0.25	0.25	0.62	0.37	0.57	0.62	**1**													
8	0.5	0.5	0.5	0.75	0.4	0.5	0.25	**1**												
9	0.6	0.6	0.57	0.8	0.5	0.57	0.5	0.6	**1**											
10	0.16	0.16	0.25	0.33	0.14	0.25	0.22	0.4	0.5	**1**										
11	0.25	0.25	0.62	0.37	0.22	0.62	0.55	0.25	0.5	0.57	**1**									
12	0.4	0.4	0.42	0.6	0.33	0.42	0.22	0.75	0.5	0.6	0.37	**1**								
13	0.25	0.25	0.62	0.37	0.22	0.62	0.55	0.42	0.5	0.57	0.75	0.57	**1**							
14	0.28	0.28	0.5	0.42	0.25	0.5	0.44	0.5	0.57	0.66	0.62	0.66	0.85	**1**						
15	0.25	0.25	0.62	0.37	0.22	0.62	0.55	0.42	0.5	0.57	0.75	0.57	**1**	0.85	**1**					
16	0.2	0.2	0.12	0.16	0.4	0.12	0.42	0.2	0.33	0.16	0.11	0.16	0.25	0.28	0.25	**1**				
17	0.4	0.4	0.42	0.6	0.33	0.42	0.37	0.75	0.8	0.6	0.37	0.6	0.57	0.66	0.57	0.4	**1**			
18	**0**	**0**	0.16	**0**	**0**	0.16	0.14	**0**	**0**	**0**	0.14	**0**	0.14	0.16	0.14	**0**	**0**	**1**		
19	**0**	**0**	0.37	0.12	0.28	0.37	0.71	0.14	0.25	0.28	0.5	0.12	0.5	0.37	0.5	0.33	0.28	0.2	**1**	
20	0.28	0.28	0.5	0.42	0.25	0.5	0.44	0.28	0.57	0.66	0.85	0.42	0.62	0.71	0.62	0.12	0.42	0.16	0.37	**1**

Diversity analysis by OPQ-17 primer (Fig. 8)

The phylogenetic tree generated by PhyElp 1.4 using the UPGMA algorithm, were clustered into two major clusters - clusters 'I' and 'II' (Fig. 9). Major cluster 'I' was divided into two sub-clusters 'Ia' and 'Ib'. Sub-cluster 'Ib' further divided into 'Ic' and 'Id'. Major cluster 'II' was simplicifolious containing B3. The sub cluster 'Ia' contained two genotypes *viz*. Z1 and B5. Subcluster 'Ic' is further divided into two clusters, comprised of seven genotypes viz. G4, B1, G3, B2, R4, Z2 and R5. Cluster 'Id' is a major sub cluster containing 10 genotypes namely R2, G5, G2,

R3 Z4, B4, Z5, Z3, G1 and R1. The Jaccard's similarity analysis, among 20 neem ecotypes exhibited genetic similarity range of 0.0 to 1.0. Maximum similarity was observed to be 1.0 between R1-B1, R3-B1, R3-R1, R4-G1, Z3-G1, Z3-R4, Z4-R2, Z5-B1, Z5-R1 and Z5-R3 while G1-B1, G4-B1, G4-G3, R1-G1, R1-G4, R2-B1, R2-G3, R2-R1, R3-G1, R3-G4, R3-R2, R4-B1, R4-R1, R4-R3, Z3-B1, Z3-R1, Z3-R3, Z4-B1, Z4-G3, Z4-R1, Z4-R3, Z5-G1, Z5-G4, Z5-R2, Z5-R4, Z5-Z3 and Z5-Z4 gave 0.87, 0.88, 0.88, 0.87, 0.88, 0.87, 0.87, 0.87, 0.87, 0.88, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.87, 0.88, 0.87, 0.87, 0.87 and 0.87 similarity respectively (Table 8). The lowest genetic similarity noted was 0.0 in B4-B3, B5-B3 and G2-B3 accession. In the principal component analysis, all the analyzed samples were separated, which is coherent with phenogram generated by UPGMA cluster analysis. The principal component explained 42.19 percent of total variation (Fig. 10).

Table 8: Jaccard's Similarity Matrix of gel banding pattern of Primer: OPQ-17

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	**1**																			
2	0.75	**1**																		
3	0.12	0.16	**1**																	
4	0.5	0.25	**0**	**1**																
5	0.25	0.33	**0**	0.5	**1**															
6	0.87	0.62	0.14	0.57	0.28	**1**														
7	0.55	0.33	**0**	0.66	0.33	0.62	**1**													
8	0.77	0.55	0.12	0.5	0.25	0.66	0.75	**1**												
9	0.88	0.66	0.11	0.44	0.22	0.77	0.66	0.88	**1**											
10	0.62	0.57	0.2	0.5	0.4	0.5	0.37	0.62	0.55	**1**										
11	**1**	0.75	0.12	0.5	0.25	0.87	0.55	0.77	0.88	0.62	**1**									
12	0.87	0.62	0.14	0.57	0.28	0.75	0.62	0.87	0.77	0.71	0.87	**1**								
13	**1**	0.75	0.12	0.5	0.25	0.87	0.55	0.77	0.88	0.62	**1**	0.87	**1**							
14	0.87	0.62	0.14	0.57	0.28	**1**	0.62	0.66	0.77	0.5	0.87	0.75	0.87	**1**						
15	0.5	0.25	0.25	0.6	0.2	0.57	0.42	0.5	0.44	0.5	0.5	0.57	0.5	0.57	**1**					
16	0.44	0.222	0.2	0.5	0.16	0.5	0.57	0.62	0.55	0.42	0.44	0.5	0.44	0.5	0.8	**1**				
17	0.37	0.28	0.33	0.4	0.25	0.42	0.28	0.37	0.33	0.6	0.37	0.42	0.37	0.42	0.75	0.6	**1**			
18	0.87	0.62	0.14	0.57	0.28	**1**	0.62	0.66	0.77	0.5	0.87	0.75	0.87	**1**	0.57	0.5	0.42	**1**		
19	0.87	0.62	0.14	0.57	0.28	0.75	0.62	0.87	0.77	0.71	0.87	**1**	0.87	0.75	0.57	0.5	0.42	0.75	**1**	
20	**1**	0.75	0.12	0.5	0.25	0.87	0.55	0.77	0.88	0.62	**1**	0.87	**1**	0.87	0.5	0.44	0.37	0.87	0.87	**1**

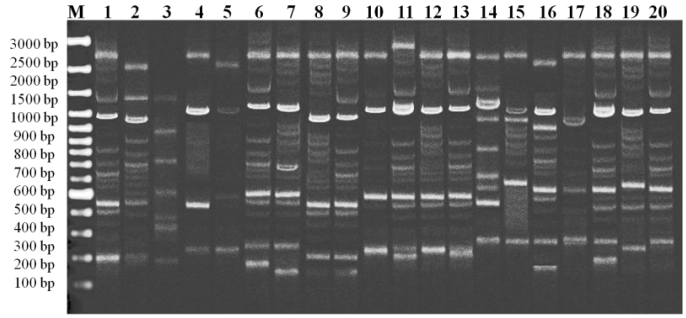

Fig. 8: RAPD banding pattern by OPQ-17 primer
Lane M: GeneRuler™ 100 bp plus ladder; Lane 1-20: Neem ecotypes

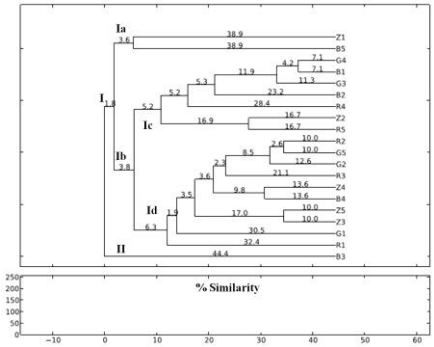

Fig. 9: Phylogenetic tree generated using PhyElp 1.4 of Primer OPQ-1

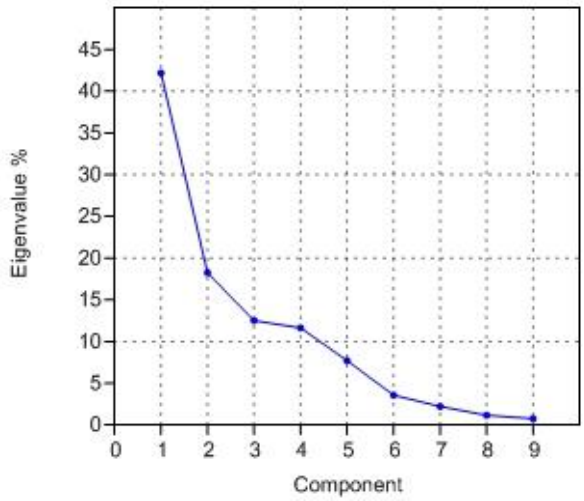

Fig. 10: Principal component analysis for primer OPQ-17

DISCUSSION

Ecological and geographical differentiations are important factors, influencing strategies for breeding and sampling tree crops. With the objective of selecting and maintaining elite trees to bring about an overall genetic improvement in neem for enhanced output of oil, provenances of neem have been collected from four regions in which three regions are from Karnataka and one is from Telangana state and are at present being maintained at Gulbarga University campus, Kalaburagi. Geographically isolated population accumulates genetic differences as they adapt to different environmental conditions (Sarwat et al., 2008). Genetic diversity analysis based on morphological and biochemical parameters has plenty of limitations due to the influence of various environmental factors. Molecular markers based genetic diversity analysis is beneficial because they are unaffected by environmental conditions.

Molecular studies using RAPD primers have been used to examine genetic variation within a species, to analyse particular genotypes, for cultivar identification and to study the clonal structure of several tree species (Kresovich et al., 1992; Vierling & Nguyen, 1992; & Hsaio & Rieseberg, 1994). High variations in the RAPD patterns have also been reported in the black and red spruce varieties, where the variation is attributed to the interaction between the plantlets and the microenvironment (Nkongolo et al., 1998). The pairwise comparison of the RAPD profiles based on both shared and unique amplification products was made to generate a similarity matrix. Similarity indices estimated on the basis of three primers ranged from 0.00 to 1.00. Such a wide range of similarity co-efficient values suggest that the neem germplasm collection represents a genetically diverse population. The similar data have been reported by Singh et al. (1999) based on their study using amplified fragment length polymorphism (AFLP). One of the major contributory factors to the high degree of polymorphism observed in neem may be on account of its evolutionary status as an out-crossing angiosperm (Kundu, 1999; & Dhillon et al., 2005). The high diversity revealed by RAPD is that out-breeding woody plants retain considerable variability (Hamrick, 1990). This was further supported by molecular marker studies (Hamrick, 1989). The maintenance of a high genetic variance within populations was favored by the genetic system of the species like effective gene flow, out breeding, mutation, high genetic load, etc (Matyas, 1996).

The RAPD study revealed that there is a genetic diversity in neem. On average, 78.94% polymorphism was detected with each RAPD fingerprint, indicating a high marker index. RAPD analysis revealed that the genetic similarity varied from a high of 100% (1.00) to a low of 0% (0.00) within the 20 neem ecotypes. Such a wide range in similarity coefficient values suggests that the neem germplasm collection represents a genetically

diverse population. One of the major contributory factors to the high degree of polymorphism observed in neem may be on account of its evolutionary status as an out-crossing angiosperm. Similar observations have been made with coconut by Perera et al. (1998) where the level of genetic diversity was shown to correlate with the breeding nature of the palms. The tall out-breeding variety exhibited a higher diversity than the inbreeding types.

Cluster analysis has clearly indicated that there are two major groups based on the similarity, values classified in neem population. Genetic relationships among neem accessions were also visualized by performing principal component analysis based on RAPD data. The principal component analysis observed on an average 37% of the total variation. This result is coherent with the dendrogram generated by employing UPGMA and is a further confirmation of the genetic similarities. Cluster analysis of the neem genotypes employing UPGMA as well as principal component analysis led to the segregation of the accessions into the distinct groups, which reflected their geographical distribution. Ecological and geographical differentiation are important factors, which influence breeding and sampling strategies of tree crops (Namkoong, 1986), which further help in understanding the population structure. The reason for the grouping of samples to one cluster collected from different sub-zones may be due to human intervention, which makes partitioning and distribution of variability complex. This assumption has been further supported by the Ranade et al. (2002), that neem has distributed throughout India from only a few groups of founder population.

Variation in genetic diversity within species is usually related with geographic range, mode of reproduction, mating systems, seed dispersal and fecundity (Loveless, 1992). Muller et al. (1992) in their review of the results of studies on genetic variation in various coniferous and angiosperm tree species observed that species with geographically distinct ranges tend to show a moderate too high-inter and intra-population genetic variation. High genetic diversity detected in the present study may be due to all these prevalent background factors as populations of CPTs studied are widely distributed in different eco-geographic regions. Similar conclusions were reached by Singh et al. (2002) while assessing genetic diversity in 37 neem accessions from different agro-climatic regions of India using AFLP and SAMPL molecular markers. They reported that neem germplasm within India constitutes a broad genetic base. The RAPD banding pattern in ecotypes of HK area also show wide genetic variation. This has resulted in fluctuation yield attributes of seeds and seed oil etc. It is necessary to identify the candidate plus trees with highly productivity genotypes and mass cultivation on wastelands for future economic and industrial development.

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REFERENCES

Cruzan, M. B. (1998). Genetic markers in plant evolutionary ecology, *Ecology*, *79*, 400-412.

Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation, *Plant. Mol. Biol. Reporter*, *1*, 19-21.

Dhillon, R. S., Bisla, S. S., & Hooda, M. S. (2005). Phenology and breeding system of neem, *Indian J Ecol*, *31*, 30-32.

Dhillon, R. S., Mohapatra, T., Singh, S., Boora, K. S., & Singh, K. (2007). Assessment of genetic diversity in *Azadirachta indica* based on DNA fingerprinting, *Indian Journal of Biotechnology*, *6*, 519-524.

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue, *Focus*, *12*, 13-15.

Hamrick, J. L., & Godt, M. J. W. (1989). Allozyme diversity in plant species, in *Plant population genetics*, Breeding and genetic resources, edited by Brown, A. H. D., Clegg, M. T.,

Kahler, A. L., & Weir, B. S. (Sinauer Assoc Sunderland, Mass) pp 43-63.

Hamrick, J. L. (1990). Isozymes and analysis of genetic structure in plant populations, in Isozymes in plant biology, edited by Soltis E D and Soltis P S (Champan and Hall, London) pp 87-105.

Hansen, M., Hallden, C., & Sall, T. (1998). Error rates and polymorphism frequencies for three RAPD protocols, *Plant Mol Biol Rep*, *16*, 139-146.

Hsaio, J. Y., & Rieseberg, L. H. (1994). Population genetic structure of *Yushania niitakayamensis* (Bambusoideae, Poaceae) in Taiwan, *Mol. Ecol*., *3*, 201-208.

Kresovich, S., Williams, J. S. K., Ferson, M. J. R., Routman, E. J., & Schaal, B. A. (1992). Characterization of genetic identities and relationships of *Brassica oleracea* L. via a Random Amplified Polymorphic DNA assay, *Theor. Appl. Genet*, *85*, 190-196.

Kundu, S. K. (1999). Comparartive analysis of seed morphometric and allozyme data among four population of neem (Azadirachta indica), *Genet Resourc Crop Evol*, *46*, 569-577.

Lodhi, M. A., Guang, N. Y., Norman, F. W., & Bruce, I. R. (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*, *Plant Mol. Biol. Rep*., *12*, 6-13.

Loveless, M. D. (1992). Isozyme variation in tropical trees, *New Forest*, *6*, 67-94.

Matyas, C. (1996). Climatic adaptation of trees: Rediscovering provenance tests, *Euphytica*, *92*, 45-54.

Muller, S. G., Baradat, P. H., & Bergmann, F. (1992). Genetic variation within European tree species, *New Forest*, *6*, 23-47.

Namkoong, G. (1986). Genetics and the forests of the future, *Unasylva*, *152*, 2-18.

Nkongolo, K. K., Klimaszewsa, K., & Gratton, W. S. (1998). DNA yields and optimization of RAPD patterns using spruce embryogenic lines, seedlings and needles, *Plant Mol. Biol. Rep*., *16*, 1-9.

Perera, L., Russell, J. R., Provan, J., McNicol, J. W., & Powell, W. (1998). Evaluating genetic relationships between indigenous coconut (*Cocos nucifera* L) accessions from Sri Lanka by means of AFLP profiling, *Theor Appl Genet*, *96*, 545-550.

Rahmatollah, B. G. S., Majid, F., Zahra, N., Jothi, M. P., & Jalaluddin, H. (2014). Effective DNA Extraction Protocol for Kenaf. *International Journal of Farming and Allied Sciences*, *3*, 103-111.

Ranade, S. A., Nuzhat, F., & Farooqui, N. (2002). Assessment of profile variations amongst provenances of neem using single primer amplification reaction (SPAR) techniques, *Molecular Biology Today*, *3*, 1-10.

Sarwat, M., Das, S., & Srivastava, P. S. (2008). Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb, *Plant Cell Rep*, *27*, 19-528.

Singh, A., Negi, M. S., Rajagopal, J., Bhatia, S., & Tomar, U. K. (1999). Assessment of genetic diversity in *Azadirachta indica* using AFLP markers, *Theoretical and Applied Genetics*, *99*, 272-279.

Vierling, R. A., & Nguyen, H. T. (1992). Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes, *Theor Appl Genet*, *84*, 835-838.

Virk, P. S., Zhu, J., Newbury, H. J., Bryan, G. J., Jackson, M. T., & Ford-Lloyd, B. V. (2000). Effectiveness of different classes of molecular markers for classifying and revealing variations in rice (Oryza sativa) germplasm, *Euphytica*, *112*, 275-284.