Morphological, Chemical and Genetic Variability in Neem Accessions

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Fifty-eight accessions of neem collected from two agro-ecological regions of North-western plains of India covering the states of Punjab, Haryana and Rajasthan wer. characterized at morphological, chemical and molecular level to ascertain the variability. For statistical treatment, the regions were sub-divided into sub-zones (categories) with the sub-zone 5 further sub-divided into two. Seed characters for morphological, oil, azadirachtin, salannin and nimbin for chemical and six AFLP primer combinations for molecular evaluation were considered. Variability was observed for all the morphological and chemical characters amongst the samples. Among the categories, significant chemical variability existed for azadirachtin-A but the morphological variability was non-significant. At molecular level the genetic similarity (Jaccard's coefficient) varied from 0.07 to 0.759. UPGMA cluster analysis grouped the accessions into five, four and three clusters, respectively based on genetic, morphological and chemical analyses. The dendrograms based on these three parameters revealed no uniformity in their grouping pattern. A high correlation between the cophenetic and dissimilarity matrices has been observed, the correlation being the highest for genetic (r = 0.969) followed by morphological (r = 0.835) and chemical (r = 0.727) matrices. Mantel's test revealed a low correlation among the three parameters.

Key words: *Azadirachta indica*, Chemical Components, Genetic Markers, Morphological Characters, Neem

Neem (*Azadirachta indica* A. Juss) is a multipurpose tree species that grows naturally in the Indian sub-continent. Of late, the tree has caught worldwide attention because of its spectacular therapeutic and bioactive properties (National Research Council, 1992; Tiwari, 1992; Schmutterer, 1995; Randhawa and Parmar, 1996;). It has a cultural heritage in India and its medicinal properties are enshrined in the ancient scriptures. A wide variation in survival, growth, morphological and physiological characters (Rajawat *et al.*, 1994; Kundu and Tigerstedt, 1997; Kundu *et al.*, 1998); physio-chemical, chemical and bioactivity parameters (Ermel *et al.*, 1987; Rengasamy *et al.*, 1993; Rengasamy and Parmar, 1995; Parmar, 1995; Kumar and Parmar 1996, 1997) and the genetic variability based on the isozyme (Kundu, 1999) and AFLP markers (Singh *et al.*, 1999) has been reported. The various studies reported variability in a narrow respect. No effort has been made to study comprehensively the morphological, chemical and genetic variability on the same population.

This paper reports variability amongst 58 accessions of neem from North-western India based on morphological and chemical characters and genetic (AFLP) markers. An attempt has been made to study the correlation amongst the different parameters by comparing their distance matrix employing Mantel test.

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Material and Methods

Neem Seeds

Fifty-eight seed samples of open-pollinated trees were collected from three states of North-western India, namely, Punjab, Haryana and Rajasthan, representing two agro-ecological regions (Fig. 1, Sehgal *et al.*, 1992) and 12 agro-climatic sub-zones (Ghosh, 1991). Details about the test accessions are given in Table 1. For genetic analysis the seeds were grown in a greenhouse of National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The samples falling in a sub-zone were put in one category except P_5, which has been divided into P_5 and $P_{5.1}$ based on aridity and rainfall in this sub-zone. Thus, a total of 13 categories formed were used for statistical analysis.

Seed Morphometric Analysis

Average seed length (mm), diameter (mm) and diameter/length (D/L) ratio were recorded based on 10 measurements each. For weight (g), hundred seeds/ accession were taken.

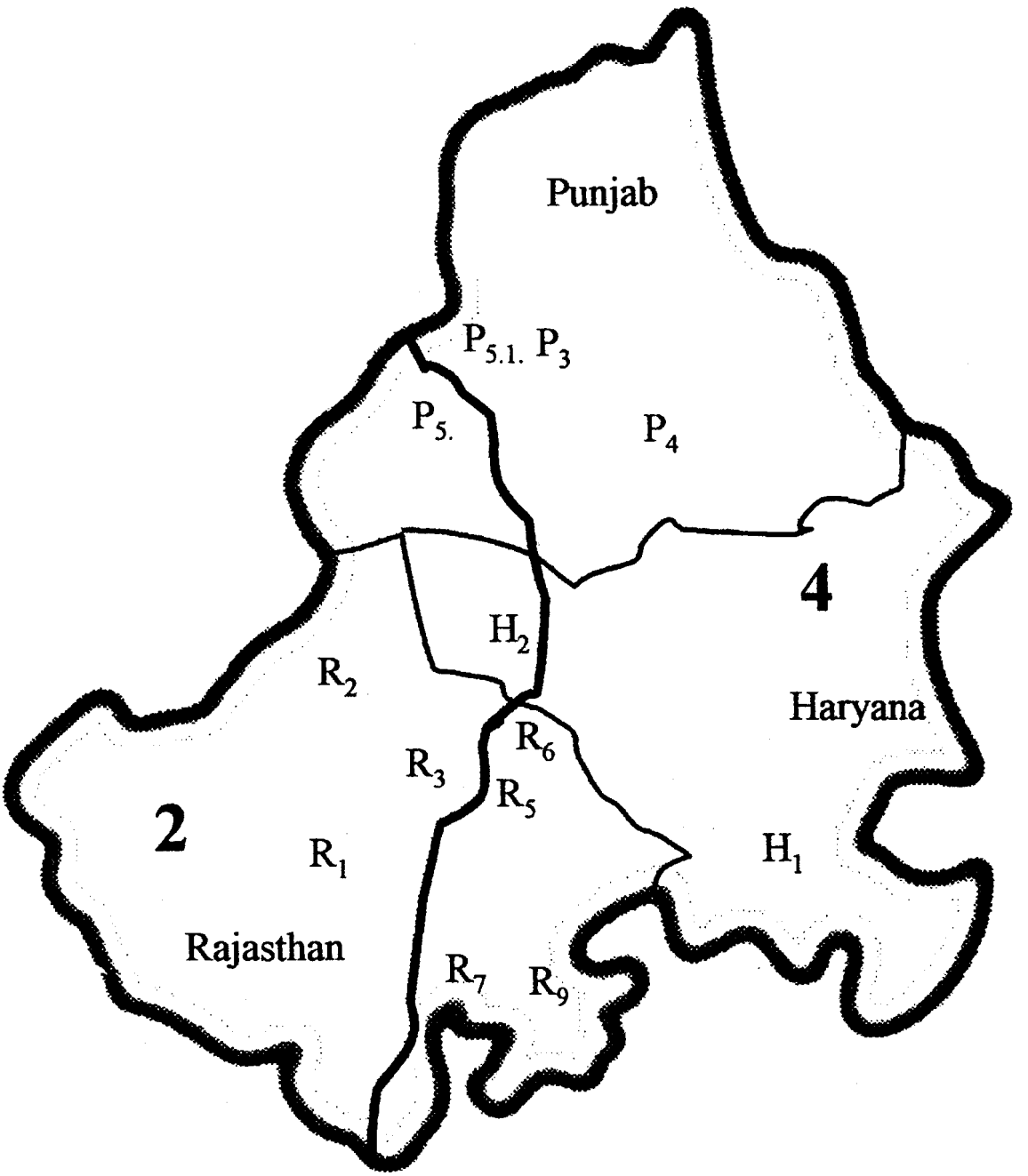

Fig. 1. Agro-climatic regions of India from where neem (Azadirachta indica A. Juss.*) samples were collected*

Chemical Parameters

Oil yield

Dry, cleaned seeds were decorticated manually to obtain kernels, which were crushed in a Waring blender. A known quantity of the crush was extracted in n-hexane (60-80 °C) in a Soxhlet extractor for 8 h (at this stage a drop of n-hexane extract when evaporated on a filter paper left no residual oily spot). n-Hexane was removed in a rotavapor under reduced pressure at 60°C to yield oil.

Table 1. Details of neem accessions used for study

Tree no.	Region	Sub-zone	Category	Tehsil, District	State
N_1	2	R_1	1	CAZRI, Jodhpur	RJ
N_2	2	R_1	1	JD-20, Jodhpur	RJ
N_3	2	R_1	1	JD-21, Jodhpur	RJ
N_4	2	R_1	1	Bhopalgarh, Jodhpur	RJ
N_5	2	R_1	1	Bhopalgarh, Jodhpur	RJ
N_6	2	R_1	1	CSWRI, Bikaner	RJ
N_7	2	R_1	1	CSWRI, Bikaner	RJ
N_8	2	R_1	1	RAU, Bikaner	RJ
N_9	2	R_1	1	RAU, Bikaner	RJ
N_{10}	2	R_2	2	Pilibanga, Bikaner	RJ
N_{11}	2	R_2	2	Ganganagar, Ganganagar	RJ
N_{12}	2	R_2	2	Ganganagar, Ganganagar	RJ
N_{13}	2	R_3	3	Khimsar, Nagor	RJ
N_{14}	2	R_3	3	Nagor, Nagor	RJ
N_{15}	2	P_5	4	Malot, Muktsar	PB
N_{16}	2	P_5	4	Malot, Muktsar	PB
N_{17}	2	P_5	4	Gidarbha, Muktsar	PB
N_{18}	2	P_5	4	Bhatinda, Bhatinda	PB
N_{19}	2	P_5	4	Bhatinda, Bhatinda	PB
N_{20}	2	P_5	4	Bhatinda, Bhatinda	PB
N_{21}	2	H_2	5	Barwala, Hisar	HR
N_{22}	2	H_2	5	CCSHAU, Hisar	HR
N_{23}	2	H_2	5	Hisar, Hisar	HR
N_{24}	2	H_2	5	Hisar, Hisar	HR
N_{25}	2	H_2	5	Mundal, Bhiwani	HR
N_{26}	2	H_2	5	Mundal, Bhiwani	HR
N_{27}	2	H_2	5	Bawal, Rewari	HR
N_{28}	2	H_2	5	Bawal, Rewari	HR
N_{29}	2	H_2	5	Bawal, Rewari	HR
N_{30}	2	H_2	5	Bawal, Rewari	HR
N_{31}	4	R_5	6	Kotputli, Jaipur	RJ
N_{32}	4	R_5	6	Amer, Jaipur	RJ
N_{33}	4	R_6	7	Bahror, Alwar	RJ
N_{34}	4	R_7	8	Jahajpur, Bhilwara	RJ
N_{35}	4	R_7	8	Jahajpur, Bhilwara	RJ
N_{47}	4	R_7	8	Chittorgarh, Chittorgarh	RJ
N_{48}	4	R_7	8	Begu, Chittorgarh	RJ
N_{49}	4	R_7	8	Begu, Chittorgarh	RJ
N_{50}	4	R_7	8	Chittorgarh, Chittorgarh	RJ
N_{36}	4	P_4	9	Barnala, Sangrur	PB
N_{37}	4	$P_{5.1}$	10	Abohar, Ferozpur	PB
N_{38}	4	$P_{5.1}$	10	Abohar, Ferozpur	PB
N_{39}	4	$P_{5.1}$	10	Abohar, Ferozpur	PB
N_{40}	4	H_1	11	Rohtak, Rohtak	HR
N_{41}	4	H_1	11	Pehwa, Rohtak	HR
N_{42}	4	H_1	11	Pehwa, Rohtak	HR
N_{43}	4	H_1	11	Kaithal, Kaithal	HR
N_{44}	4	H_1	11	Kaithal, Kaithal	HR
N_{45}	4	H_1	11	Narwana, Jind	HR
N_{46}	4	H_1	11	Rohtak, Rohtak	HR
N_{51}	4	R_9	12	Hindoli, Bundi	HR
N_{52}	4	R_9	12	Bundi, Bundi	RJ
N_{53}	4	R_9	12	Ladpura, Kota	RJ
N_{54}	4	P_3	13	Jagroan, Ludiana	PB
N_{55}	4	P_3	13	PAU, Ludhiana	PB
N_{56}	4	P_3	13	Khanna, Ludhiana	PB
N_{57}	4	P_3	13	Amoth, Fatejgarh Sahib	PB
N_{58}	4	P_3	13	Rajpura, Rajpura	PB

RJ = Rajasthan, PB = Punjab, HR = Haryana
PAU = Punjab Agricultural University, RAU = Rajasthan Agricultural University
CCSHAU = Chaudhary Charan Singh Haryana Agricultural University, JD = Jodhpur
CAZRI = Central Arid Zone Research Institute
CSWRI = Central Soil and Water Conservation Research Institute
$P_{5.1}$ = Classified based on aridity and rainfall of the sub-zone.

Azadirachtin, salannin and nimbin content

Five gram lots of each of the crushed samples (3 replicates each) were immersed in 10 ml methanol and agitated in an ultrasonic bath for 5 min. The contents were filtered through a G2 Buchner funnel. The residue was re-extracted twice, each time with 7 ml methanol and filtered. The filtrates were combined and volume made to 25 ml. Two ml of this solution was cleaned up by passing through Lichrolut filter cartridge containing RP-18 material and analyzed for azadirachtin, salannin and nimbin contents employing Shimadzu HPLC system fitted with LC9A pumps in binary mode, a Rheodyne 7161 injector with a 20 ml loop and a SPDM6A photodiode array detector. Samples were resolved isocratically on a 15 cm x 6 mm i.d. Shimpack CLC phenyl stainless steel column using methanol-water (70:30) mobile phase at 1.0 ml min^{-1}. Absorbance was measured at 214 and 250 nm at sensitivity of 0.05 AUFS. Each chromatogram was run up to 20 min. The data were acquired on a PCS-DG India Ltd. Work station and quantification was done in the post-analysis session at 214 nm. Under these conditions, azadirachtin, nimbin and salannin showed retention times of 7.13, 13.43 and 15.36 min, respectively.

Genetic Analysis

DNA extraction

Total genomic DNA was isolated from the leaves of 8-week-old seedlings by the method of Dellaporta *et al.* (1983). DNA was quantified using DyNA Quant Fluorometer (Hoefer) and samples were diluted to a final concentration of 100 ng/ml.

AFLP analysis

AFLP™ Plant Mapping Kit (Perkin Elmer Biosystems Inc., USA) was used for DNA restriction, ligation, pre-selective and selective amplification. The amplification reactions were carried out on Perkin-Elmer DNA thermal cycler 9600. The selective amplification reactions were denatured with ABI GENESCAN 500 ROX internal standard for 3 min. at 93°C and chilled quickly by placing on ice before loading. The primer combinations used during selective amplification are given in Table 3. DNA samples were capillary electrophoresed at constant voltage (14.9 KV) and current (12.9 mA) at a temperature of 60°C on an automated DNA sequencer (PE Applied Biosystems model ABI Prism 310) equipped with Genescan software. Analysis of data was done with Genescan analysis software.

Data Analyses

Seed morphometric and chemical data

The data were analyzed for the differences amongst the categories by analysis of variance (ANOVA) using PROC GLM procedure of the SAS statistical software package (SAS Institute Inc., Cary, NC, USA). When differences were significant the means were separated by Duncan's Multiple Range Test (P = 0.06). Contrast analysis was also carried out to test the equality of region means and categories within the regions. For this CONTRAST statement, PROC GLM of SAS was used. Morphological and chemical data were also subjected to interval analysis using NTSYS Software (version 1.70). Distance coefficients were used to generate the dendrogram employing UPGMA (Unweighted pair grouping method of average, Sneath and Sokal, 1973). In addition, principal co-ordinate analysis was performed.

Genetic data

Binary data obtained in the form of peaks during analyses were converted to numerical values using Genotyper (version 2.5). AFLP bands ranging in size from 50 to 500 bp (with minimum peak height 100) were scored for presence (1) and absence (0) across the neem accessions of each primer combination. The pair-wise genetic similarity among the samples was estimated according to the Jaccard's coefficient. The statistical analysis was carried using NTSYS software (version 1.70). The similarity values were converted to distance (D) by subtracting each value from unity. A dendrogram was constructed by employing UPGMA in order to group genotypes into discrete clusters. In addition, principal co-ordinate analysis was performed. Resolving power for each primer has been calculated. According to Prevost and Wilkinson (1999) resolving power (Rp) of a primer is: Rp = Ib, where Ib (band informativeness) takes value for each primer and has been calculated as $1-|2'(0.5-p)|$, p being the proportion of the 58 accessions containing the bands.

SPSS software package was used for the squared Euclidean distance analysis (as this was the only distance criteria which was common for both qualitative and quantitative data) for all three parameters and the average linkage (between samples) was used for the construction of dendrogram on uniform scale.

Matrix comparison

The Mantel matrix correspondence test (Mantel, 1967) was used to compare the cophenetic matrices with similarity matrices to define the degree of congruence as well as to compare similarity and cophenetic matrix among the three parameters to establish the level of association between them.

Results

Seed Morphometric Parameters

All the characters, namely, seed length (9.8-16.5 mm), seed diameter (4.9-7.8 mm), D/L ratio (0.38-0.63) and 10-seed weight (9.8-26.21g) showed variation. Weighted mean and standard deviation for each character from the two regions are reported in Table 2. Contrast analysis revealed that there was no significant difference between the regions for all the seed parameters. However, seed diameter showed variation among the categories of the region 4 at 6% level of significance (Table 3). No significant difference has been found when comparison was done at the level of categories (Table 2).

Chemical Parameters

Contrast analysis revealed significant differences between the regions for oil yield. However, differences were non-significant for other characters (Table 3). The categories of region 4 were found to be significantly different with respect to oil yield and azadirachtin (Table 3). At category level (Table 2), the azadirachtin varied significantly (P = 0.06) from 0.21-0.57. For azadirachtin P_3 sub-region (Punjab) showed the maximum variation followed by H_1 sub-region (Haryana). The oil, salannin and nimbin contents varied non-significantly for all 13 categories. Oil yield varied from 27.16-56.11, salannin 0.03-1.09 and nimbin 0.13-1.19% (w/w).

Fingerprint Profile

The 58 accessions of neem using six selective AFLP primer combinations (Table 4) generated a total of 567 amplicons with an average of 94.5 amplicons/primer. All the primers were polymorphic and generated accession specific bands. The primer combinations MseI-CTG/EcoRI-ACT, MseI-CTG/EcoRI-ACC, MseI-CTG/EcoRI-ACG were more informative as these generated more than hundred bands. Each combination MseI-CTG/EcoRI-ACT showed the highest (35.38) and MseI-CTT/EcoRI-AAC, the least (2.76) resolving power (Table 4). Data

Table 2. Region- and category- wise means of morphological and chemical data on the neem accessions

Region	Category	No. g samples	Seed length (mm)	Seed diameter (mm)	D/L ratio	100-seed weight	Oil yield (% w/w)	Azadirachtin (%0	Salannin (% w/w)	Nimbin (% w/w)
2	1	9	13.26	6.46	0.49	17.96	42.61	$0.472^{b,c}$	0.313	0.551
	2	3	14.20	6.20	0.43	17.17	45.15	$0.453^{b,c}$	0.383	0.547
	3	2	12.10	6.45	0.53	15.46	47.92	$0.450^{b,a,c}$	0.445	0.470
	4	6	12.32	6.57	0.54	16.51	44.43	$0.393^{b,c}$	0.370	0.407
	5	10	13.26	6.56	0.49	17.31	43.03	$0.494^{b,c}$	0.288	0.535
Weighted Mean		30	13.09	6.49	0.50	17.21	43.81	0.460	0.332	0.511
SD ±			1.52	0.49	0.06	3.41	4.60	0.09	0.18	0.27
4	6	2	13.30	6.05	0.45	13.86	49.58	$0.260^{b,c}$	0.305	0.385
	7	1	11.70	4.90	0.41	9.80	38.66	0.210^{c}	0.230	0.380
	8	6	13.13	6.70	0.51	16.76	42.25	$0.331^{b,a,c}$	0.413	0.450
	9	1	13.40	6.90	0.51	20.50	27.16	0.520^{a}	0.760	0.530
	10	3	12.97	6.66	0.52	16.95	45.17	$0.443^{b,a}$	0.613	0.633
	11	7	13.81	6.77	0.49	18.07	44.27	$0.527^{b,c}$	0.329	0.459
	12	3	13.60	6.40	0.47	17.87	40.80	$0.360^{b,c}$	0.326	0.327
	13	5	14.72	6.74	0.46	15.57	43.62	$0.578^{b,a,c}$	0.566	0.842
Weighted Mean		28	13.59	6.59	0.48	16.69	43.01	0.437	0.430	0.556
SD ±			1.26	0.59	0.05	4.41	5.75	0.19	0.22	0.23
			NS	NS	NS	NS	NS	S	NS	NS

Means with the same superscript letters are not significantly different at P = 0.06 using DMRT (Duncan's Multiple Range Test)
NS = Nonsignificant S = Significant

Table 3. Significance level of equality of region means, category means within region

Contrast	Seed	Seed length (mm)	D/L ratio diameter	100 seed weight (g)	Oil yield (% w/w)	Azadirachtin (% w/w)	Salannin (% w/w)	Nimbin (% w/w)
Region	0.51	0.74	0.29	0.59	0.04*	0.27	0.19	0.66
Categories (region 2)	0.29	0.85	0.11	0.93	0.65	0.69	0.78	0.83
Categories (region 4)	0.47	0.05*	0.48	0.53	0.03*	0.01*	0.09	0.18

*Significant at P = 0.06

were used to make pair-wise comparison of the lines based on shared and unique amplification products to generate similarity matrix using the NTSYS-PC statistical package (Version 1.70) The value of similarity for all the 58 lines ranged from 0.07 to 0.759. The two neem samples N_1 and N_{50}, collected from Jodhpur (sub-zone R_1, Rajasthan) and Chittorgarh (sub-zone R_7, Rajasthan), respectively, displayed greater genetic similarity with a similarity coefficient of 0.759.

Cluster and Principal Coordinate Analysis

Morphological clusters

Cluster analysis of the morphological data yielded a phenogram (Fig. 2), which grouped the 58 accessions into four clusters. N_{58} was distinct from all other samples. The principal coordinate analysis revealed that the first three coordinates explained 88.24, 10.55 and 1.79% of total variation.

Chemical clusters

Cluster analysis of the chemical data gave the resultant phenogram (Fig. 3), which grouped the 58 accessions into three clusters. The principal coordinate analysis revealed that the first three coordinates explained 80.75, 18.92 and 0.92% of total variation.

Genetic clusters

Cluster analysis of the genetic data yielded phenogram (Fig. 4), which grouped the 58 accessions broadly into five major clusters based on similarity coefficient. Except clusters 1 and 2, the other clusters are represented by two samples each. Clusters 1 and 2 are represented by samples from all the three states. Therefore, no geographical isolation has been observed. Cluster 1 was further sub- divided into four sub-clusters (a-d). Cluster 1d as represented by the samples of category 11 and 13 has the highest azadirachtin variability. In cluster 5, two samples collected from Begu (Rajasthan) are grouping together and are very distinct from the other samples. In principal coordinate analysis the first three principal coordinates explained 55.98% of the total variation. In this analysis, all the analysed samples were separated which is coherent with the phenogram generated by UPGMA cluster analysis.

Squared Euclidean Distance Analysis was also performed using average linkage between groups. Six clusters were prepared based on the hierarchical analysis. In morphological dendrogram (dendrogram not presented here), N_{42} and N_{47} were distinct from rest of the samples and in chemical analysis N_2 and N_{37} were distinct from the rest. Dendrogram based on binary data showed that N_{46} and N_{55} are distinct from the rest of the accessions. Hence, no uniformity was observed between the dendrograms.

Table 4. Selective AFLP primer combinations along with total number of bands amplified and resolving power (Rp)

Mse I primers/ EcoRI primers	Total no. bands amplified	Resolving power (R_p)
MseI-CTG/EcoRI-ACT	120	35.38
MseI-CTG/EcoRI-ACC	123	33.83
MseI-CTG/EcoRI-ACG	109	18.69
MseI-CTT/EcoRI-ACA	90	9.20
MseI-CTT/EcoRI-AAC	55	2.76
MseI-CTT/EcoRI-AGG	70	8.41

Comparison of cluster and matrices

Comparison of the three dendrograms for the grouping pattern revealed no uniformity. But high correlation between the cophenetic and dissimilarity matrices for each of the three parameters was observed and this correlation was the highest for genetic matrix (r = 0.969) followed by morphological matrix (r = 0.835) and chemical matrix (r = 0.727). The correlation between the dissimilarity matrices of the three parameters was also tested using the Mantel's test. A low correlation was observed between the marker systems studied. A relatively high correlation (r = 0.16) was observed between morphological and genetic matrices, whereas chemical and morphological matrices were weakly

Fig. 2. Dendrogram generated by UPGMA based on morphological characters of 58 accessions of neem (Azadirachta indica)

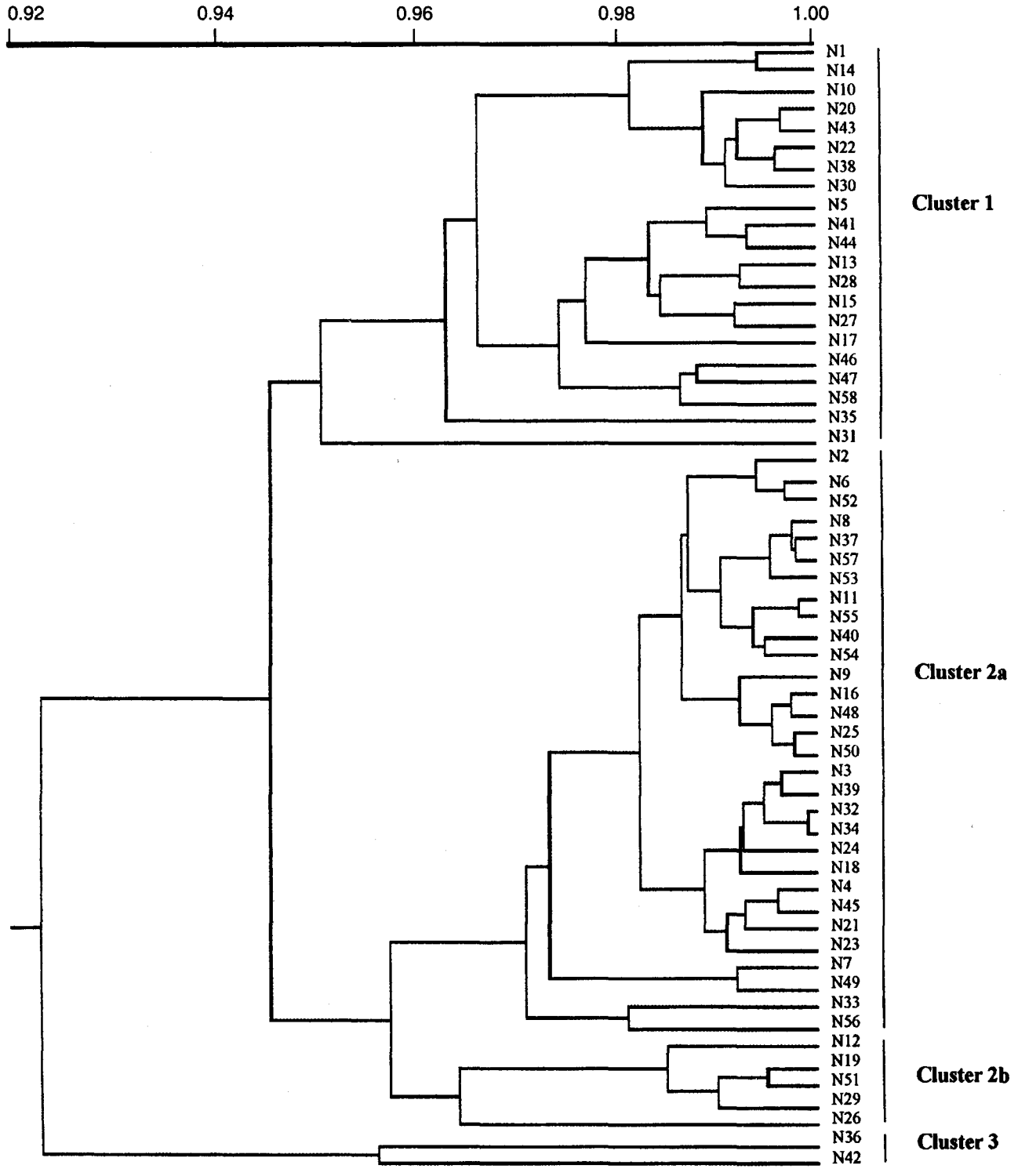

Fig. 3. Dendrogram generated by UPGMA based on chemical characters of 58 accessions of neem (Azadirachta indica)

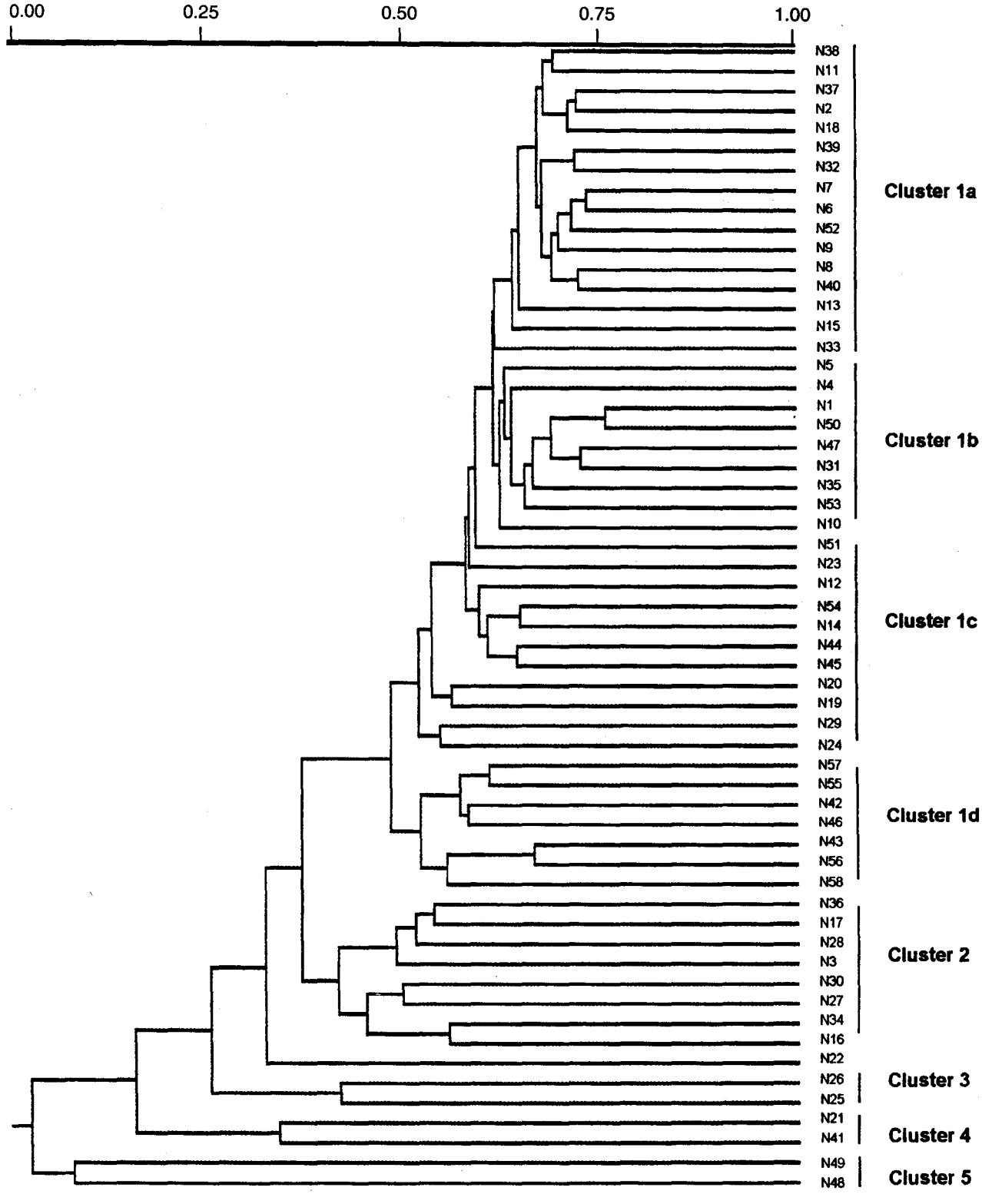

Fig. 4. Dendrogram generated by UPGMA based on Jaccard's coefficients of AFLP markers for 58 accessions of neem (Azadirachta indica)

correlated (0.0068). However, when cophenetic matrices of these marker systems were compared with each other, the correlation values were almost similar ranging from 0.0027 to 0.012.

Discussion

Objective of this study was to estimate the diversity existing in neem in the North-western region of India, and to establish the relationship between morphological, chemical and genetic data to enable selection of some elite lines to bring about an over all improvement in neem for enhanced output of oil, azadirachtin, nimbin and salannin. A high degree of genetic polymorphism and variation in the amount of azadirachtin was detected in all the categories of neem accessions selected for the study. No seed morphometric variation was detected at category level. However, when significance level of equality of region mean was tested, seed diameter showed variation for the region 4. Kundu (1999) reported significant variation in morphological data based on a study of four different neem populations collected from different countries. In the present study, no significant variation in morphology was observed, probably because the population represented a limited North-western part of India. Azadirachtin content showed significant variation for the sub-zone P_5 (Punjab) followed by H_1 (Haryana). This clearly shows that within a population, much variation existed for azadirachtin. Amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) has been widely used for differentiating between closely related cultivars in different crop species and to detect genetic variability in the germplasm of a crop species. The technique permits analysis of large number of loci in a single experiment and detects more polymorphism as compared to RFLP and RAPD methods (Powell *et al.*, 1996). The present genetic diversity estimated by AFLP is very close to that reported by Kundu (1999) based on isozyme analysis. Among the primer combinations used in the present study, three out of six primer combinations produced more than one hundred bands (Table 4). Such a large number of polymorphic fragments generated with specific primer pairs has also been reported in biomass willows (Barker *et al.*, 1999) and daylily (Tomkins *et al.*, 2001). Ellis *et al.* (1997) suggested that most informative primer combinations have the greatest coverage across the genome. The three primer combinations have apparently covered widely the genome and can be useful markers for fingerprinting work (Table 4). However, the principal component analysis has shown that the first three components could explain only 55.98% of the variability. Therefore, to cover the genome more extensively, there is need to study the variability with more numbers of AFLP primers as well as different types of marker systems such as simple sequence repeats. A linear correlation was found between resolving power and ability of primer to distinguish the accessions of neem. Thus, primer combination Mse I CTG/EcoR I ACT (Rp = 35.83) and Mse I CTG/ EcoR I ACC (Rp = 33.38) were able to distinguish all the 58 accessions. The result of cluster analysis of seed morphometric, chemical and genetic data presented different pictures. A low correlation between different parameters was observed on the basis of comparison of cophenetic and similarity matrices using Mantel's matrix correspondence test. The poor correlation between genetic similarity value based on AFLP markers and morphological characters may be caused by marker sampling error and biased representation of genome differences revealed by AFLPs (Schut *et al.*, 1997). Little or no consistency between morphological and genetic distances should not be considered a limitation of this system. Morphologically similar genotype may not be genotypically similar, as different genepool can be manipulated to create similar phenotypes. Close association, on the other hand, between morphological and genetic relationships would indicate a very restricted commercial genepool. Similarly, weak correlation between chemical and genetic matrices can be explained, because for a chemical synthesis usually a pathway consisting of several enzymes (more than one gene) plays their role. A weak correlation between morphological and chemical data suggests that seed morphometry has no relation with azadirachtin content. The low correlation between the genetic parameter with chemical or morphology is expected because all the loci, which are actually responsible for chemical and morphological gene expression, are not being covered by the genetic markers. And marker used here is mostly repeated sequence. To establish better correlation, there is need to use different DNA marker systems such as those based on ESTs, so that maximum number of functional loci may be covered and better relation among the three parameters will be established to enable supporting the future neem tree improvement programme.

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