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Assessment of genetic diversity in *Azadirachta indica* using AFLP markers

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Abstract Genetic diversity was estimated in 37 neem accessions from different eco-geographic regions of India and four exotic lines from Thailand using AFLP markers. Seven AFLP selective primer combinations generated a total of 422 amplification products. The average number of scorable fragments was 60 per experiment, and a high degree (69.8%) of polymorphism was obtained per assay with values ranging from 58% to 83.8%. Several rare and accession-specific bands were identified which could be effectively used to distinguish the different genotypes. Genetic relationships within the accessions were evaluated by generating a similarity matrix based on the Jaccard index. The phenetic dendrogram generated by UPGMA as well as principal correspondence analysis separated the 37 Indian genotypes from the four Thai lines. The cluster analysis indicated that neem germplasm within India constitutes a broad genetic base with the values of genetic similarity coefficient ranging from 0.74 to 0.93. Also, the Indian genotypes were more dispersed on the principal correspondence plot, indicating a wide genetic base. The four lines from Thailand, on the other hand, formed a narrow genetic base with similarity coefficients ranging from 0.88 to 0.92. The lowest genetic similarity coefficient value (0.47) was observed between an Indian and an exotic genotype. The level of genetic variation detected within the neem accessions with AFLP analysis suggests that it is an efficient marker technology for delineating genetic relationships amongst genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategies for conservation and tree improvement programs.

Key words Neem · AFLP · Genetic diversity

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Introduction

Azadirachta indica A. Juss., or neem, is a multipurpose tropical tree belonging to the family Meliaceae. The species is of commercial importance, primarily due to its medicinal and biopesticidal properties. It is of special cultural significance as its medicinal and pesticidal uses are mentioned in the ancient scripts of India. The important active ingredients, azadirachtin and salanin, are derived in high concentrations from the seeds. Neem-based products such as Azatin®, Turplex®, Align® and Margosan® have been introduced as insecticides in USA markets (Board on Science and Technology for International Development 1992). Neem is a native of India and Burma and has been introduced into North America and several African countries. It plays a major role in preventing soil erosion and desertification and has therefore been planted in countries like Somalia and Mauritiana to curb the southwards spread of the Sahara (Board on Science and Technology for International Development 1992). Neem grows prolifically across the Indian subcontinent. In India, neem occurs in tropical dry deciduous and thorn forests (Tewari 1992) and in drier parts up to an altitude of 1500 m. The tree is endowed with a high degree of edaphic and climatic adaptability. It can grow in different soil types and in areas with a mean annual temperature ranging from 10° to 45°C and rainfall of 450 to 1150 mm. The wide geographical and climatic distribution is indicative of the fact that there exists

a tremendous genetic diversity in neem which needs to be identified and catalogued.

A large number of methodologies exist for the assessment of genetic diversity in plant species. A combination of morphological traits and protein profiling methods such as isozymes (Nevo et al. 1986), allozymes (May 1992) and seed storage proteins (Doll and Brown 1979) have conventionally been applied. However, such traits are influenced by environmental factors as well as the developmental stage of the plants. Hence, the results elucidated based on such studies do not provide a true measure of the genetic diversity.

DNA-based markers provide useful information regarding genetic diversity and relationships between accessions as these remain unaffected by the aforementioned factors. Of the various kinds of DNA-based markers characterized so far, restriction fragment length polymorphisms (RFLPs) were the first to provide the means to directly detect variations present at the DNA level. RFLPs have been used to document genetic diversity in cultivated plants and their wild relatives (Tanksley et al. 1989; Wang et al. 1992; Diers and Osborn 1994). Although highly specific, performing RFLPs is quite tedious and expensive since it requires large amounts of pure quality DNA and an expertise in handling radioactivity. Randomly amplified polymorphic DNAs (RAPDs) (Welsh and McClelland 1990; Williams et al. 1990), a polymerase chain reaction (PCR)-based technique, resolved most of the technical obstacles owing to its cost-effective and easy-to-perform approach. This efficient technique obviates the need to work with radioisotopes and gives satisfactory results even with crude DNA preparations. RAPDs have therefore been extensively used in assessing genetic relationships amongst various accessions of different plant species (Chalmers et al. 1992; Adams et al. 1993; Castiglione et al. 1993; Russell et al. 1993; Wachira et al. 1995). One of the major drawbacks of RAPDs, however, is the lack of specificity and reproducibility. It has been observed that RAPD profiles are highly sensitive to variations in the concentrations of template DNA (Davin-Regli et al. 1995), Mg^{2+} ions, *Taq* polymerase and thermal cycler used. Thus, the results obtained through RAPDs can be arbitrary.

The introduction of amplified fragment length polymorpism (AFLP) as a technique for precision genotyping circumvents all the limitations of previous fingerprinting techniques (Zabeau and Vos 1993; Vos et al. 1995). The technique is highly specific, generates a high multiplex ratio and is repeatable. AFLP methodology has been used to assess genetic diversity in *Lactuca* (Hill et al. 1996), soybean (Maughan et al. 1996), *Lens* (Sharma et al. 1996), sunflower (Hongtrakul et al. 1997), tea (Paul et al. 1997) and barley (Russell et al. 1997). Biodiversity in rice, hops and grapevine was successfully analyzed by Zhu et al. (1998), Hartl and Seefelder (1998) and Cervera et al. (1998), respectively, using AFLP. Tohme et al. (1996) studied the gene pool in wild bean core collection with the aid of AFLP.

To the best of our knowledge, there has been no report on the extent of the genetic diversity prevalent in *Azadirachta indica* A. Juss (neem) species despite its manifold uses and wide distribution. Hence, the present study was undertaken to assess the genetic diversity in neem from India and Thailand using AFLP methodology. We presume that the process of naturalization of neem in diverse eco-geographic backgrounds in India is due to successful adaptations and the accumulation of useful variations in the genome during the course of evolution. We report here the assessment of genetic relationships between 41 accessions of neem from India and Thailand using AFLP markers.

Materials and methods

Plant material

The main objective in selecting for neem lines was to identify suitable provenances with desirable traits or ones yielding higher levels of active ingredients. Leaf material was collected from the field station of the Arid Forest Research Institute (AFRI) at Jodhpur, where a provenance trial project coordinated globally by FAO and other International Institutes has been launched (Chowdhry and Emmanual 1995). Seeds were collected from 37 different provenances representing ten states of India taking care that plants from every agro-climatic zone of India were incorporated in the germplasm collection. Four exotics from Thailand, namely *Azadirachta indica* var 'siamensis Valenton', which were also the provenances selected for an international trial by the Royal Forest Department (RFD) of Thailand after surveying and documenting 39 provenances (Boonchoob 1995), were used in the study. A comprehensive list of the genotypes used in this study along with their latitudinal and longitudinal positions is tabulated in Table 1.

AFLP analysis

DNA was extracted from lyophilized leaf material using a modified CTAB method of Doyle and Doyle (1990). AFLP fingerprints were generated based on the protocol of Zabeau and Vos (1993) with some modifications. Genomic DNA (250 ng) was restricted with *Eco*RI and *Mse*I (1.25 U/µl each) in a restriction buffer (50 m*M* TRIS-HCl, pH-7.5, 50 m*M* Mg acetate, 250 m*M* K acetate) in a total volume of 25 µl. *Mse*I and *Eco*RI adapters were subsequently ligated to digested DNA fragments. The adapter-ligated DNA was pre-amplified using the following cycling parameters: 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The pre-amplified DNA was diluted in a ratio of 1 : 50 and was used as a template for the selective amplification which involved the use of +3 primers (*Eco*RI and *Mse*I). The cycling parameters were: 1 cycle of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. The annealing temperature was lowered by 0.7°C per cycle during the first 12 cycles, and then 23 cycles were performed at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. The samples were resolved on 5% polyacrylamide gel and autoradiographed (Sambrook et al. 1989).

Data analysis

AFLP bands ranging in size from 50 to 350 bp were scored for absence (0) or presence (1) across the 41 neem lines for each primer

Table 1 Longitudinal and latitudinal positions of the regions from where the 41 neem accessions were collected. The first column corresponds to the code assigned to the accession number

Name of accession	Region	Geographical location
A	Gurgaon	77.1E, 28.5N
B	Pali	73.3E, 25.8N
C	Indore	75.9E, 22.7N
D	Kanpur	86.4E, 26.5N
E	Raipur	81.6E, 21.2N
F	Ujjain	75.8E, 23.2N
G	Sikar	75.1E, 27.6N
H	Jodhpur	73E, 26.3N
I	Bankura	87.1E, 23.2N
K	New Delhi	77.2E, 28.6N
L	Jaisalmer	70.9E, 26.83N
M	Mathura	77.7E, 27.5N
N	Kothalia (Rewa)	81.3E, 24.5N
O	Shivpuri	77.6E, 25.4N
P	Nagpur	73.7E, 27.2N
Q	Muzzafarnagar	85.4E, 26.1N
R	Kanpur	86.4E, 26.5N
S	Sholapur	75.8E, 17.7N
T	Khao kuang	99.57E, 15.32N
U	Ban Huey Sein	99.59E, 12.41N
V	Jhansi	78.6E, 25.4N
W	Pune	73.9E, 18.5N
X	Bikaner	73.3E, 28.1N
Y	Rajkot	70.8E, 22.3N
Z	Gandhi Nagar	76.6E, 23N
A1	Palampur	72.4E, 24.2N
A2	Indore	75.9E, 22.7N
A3	Kanpur	86.4E, 26.5N
A4	Sohangi Rewa	81.3E, 24.5N
A5	Kanpur	86.4E, 26.5N
A6	Satna	80.8E, 24.6N
A7	Mehekar	76.6E, 20.2N
A8	Bilaspur	82.2E, 22.1N
A9	Ranchi	85.3E, 23.4N
A10	Sawai Madhopur	76.4E, 26N
A11	Haunshgabad	77.7E, 22.8N
A12	Jabalpur	79.9E, 23.2N
A14	Doi Tan	98.44E, 17.57N
J	Ravinagar	
A13	Mlug	
A15	Uthaithani	

combination. The genetic similarity (GS) between pairs was estimated according to the Jaccard coefficient [Jaccard 1908; $GS(ij) = 2a/(2a + b + c)$], where GS(ij) is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j and c is the number of bands present in j and absent in i. The statistical analysis was carried out using the NTSYS-pc software (version 1.5, Rohlf 1989). A dendrogram was constructed by employing UPGMA (unweighted pair grouping method of averages; Sneath and Sokal, 1973) in order to group genotypes into discrete clusters. In addition, principal correspondence analysis was also performed.

Results

AFLP profile analysis

AFLP was used to assay 41 neem genotypes using seven *Eco*RI and *Mse*I selective primer combinations. Sequences of oligonucleotide adapters and primers used in this study are given in Table 2. A total of 422 fragments were obtained of which 297 (69.8%) were polymorphic across the Indian as well as exotic genotypes. A representative AFLP profile obtained with primer combination $E_{AAC} \times M_{CTG}$, (Fig. 1) is given for 22 neem genotypes comprising 20 Indian accessions and two exotic genotypes belonging to Thailand. A total of 54 scorable amplification products were clearly visible with this primer combination, and the overall percent polymorphism was 70.3%. However, when the exotics were excluded, only 35% polymorphism was observed within the Indian genotypes. Some bands, marked 1 and 6 (Fig. 1), were found to be monomorphic across all the genotypes. In contrast, bands that could distinguish clearly between the Indian and exotic genotypes were also observed, such as band 3 (Fig. 1), which was found to be monomorphic across Indian cultivars and absent in the Thai genotypes. In addition, bands present only in Thailand lines Khao kuang and Ban Huey Sein (band 2 and 7, Fig. 1) were also identified. Polymorphic bands 4, 5, and 8 (Fig. 1) were considered rare since they were present in fewer than 20%

Table 2 Sequences of oligonucleotide adapters and primers used in pre-amplification and seven selective AFLP primer combinations

Name	Code	Sequence
*Eco*RI adapter		5′-AAT TGG TAC GCA GTC TAC-3′ 3′-CC ATG CGT CAG ATG CTC-5′
*Mse*I adapter		5′-TAC TCA GGA CTC AT-3′ 3′-G AGT CCT GAG TAG CAG-5′
*Eco*RI + 1 primer	E_A	5′-GAC TGC GTA CCA ATT C**A**-3′
*Mse*I + 1 primer	M_C	5′-GAT GAG TCC TGA GTA A**C**-3′
*Eco*RI + 3-AAC	E_{AAC}	5′-GAC TGC GTA CCA ATT C**AAC**-3′
*Eco*RI + 3-ACA	E_{ACA}	5′-GAC TGC GTA CCA ATT C**ACA**-3′
*Eco*RI + 3-AAG	E_{AAG}	5′-GAC TGC GTA CCA ATT C **AAG**-3′
*Eco*RI + 3-ACG	E_{ACG}	5′-GAC TGC GTA CCA ATT C **ACG**-3′
*Mse*I + 3-CAT	M_{CAT}	5′-GAC TGC GTA CCA ATT C **CAT**-3′
*Mse*I + 3-CTG	M_{CTG}	5′-GAC TGC GTA CCA ATT C **CTG**-3′
*Mse*I + 3-CTA	M_{CTA}	5′-GAC TGC GTA CCA ATT C **CTA**-3′
*Mse*I + 3-CAC	M_{CAC}	5′-GAC TGC GTA CCA ATT C **CAC**-3′
*Mse*I + 3-CAG	M_{CAG}	5′-GAC TGC GTA CCA ATT C **CAG**-3′

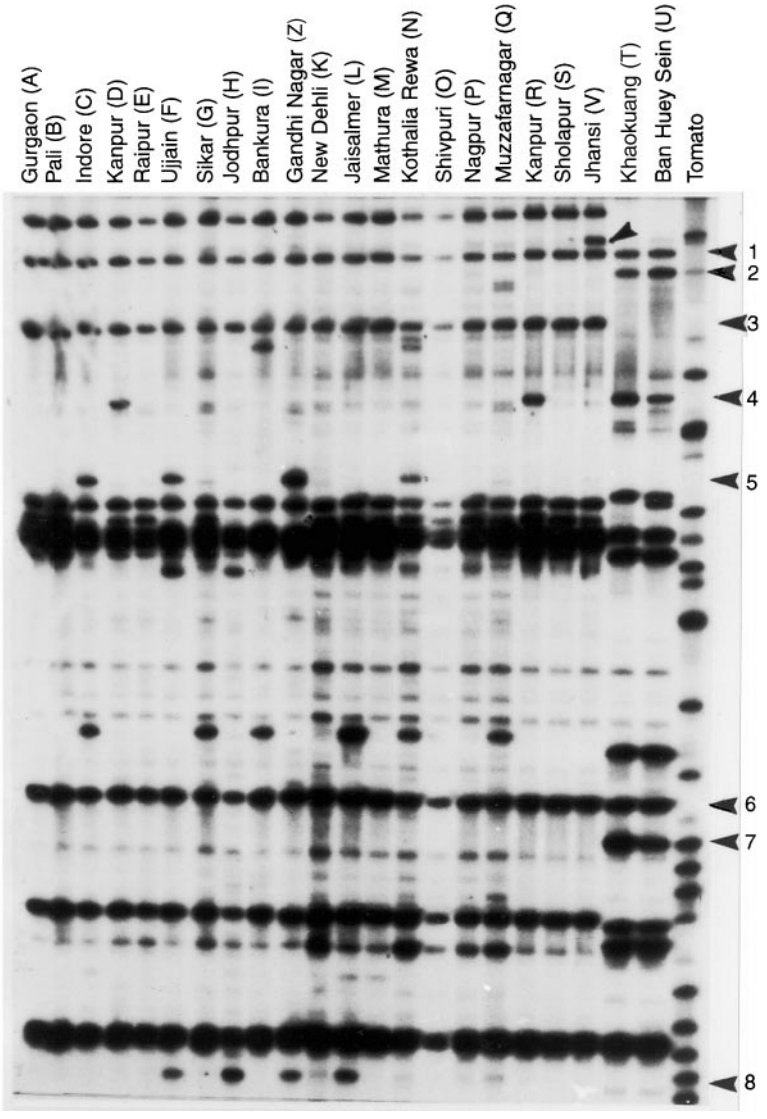

Fig. 1 AFLP analysis of *Azadirachta indica*. The AFLP fingerprints were generated using primer combination $E_{AAC} \times M_{CTG}$ to amplify genomic fragments from 22 genotypes of neem representing germplasm from different geographical regions of India. Lanes marked as *T* and *U* are the exotic varieties from Thailand. Tomato was used as a control. *Arrowheads 1* and *6* indicate monomorphic bands across all genotypes, *arrowhead 3* monomorphic bands across Indian cultivars that were absent in the Thai genotypes, *arrowheads 2* and *7* bands present in two of the Thai genotypes only, *arrowheads 4*, *5*, *8* polymorphic bands. The band indicated in the *Jhansi lane* (*V*) (*arrowhead*) is genotype-specific

of the accessions. Using this primer combination two accession-specific bands were identified. One such product was present in the genotype collected from Jhansi (the band shown by an arrowhead, Fig. 1). Such bands can be converted into accession-specific PCR markers, which may be used for the identification of accessions.

Figure 2 represents the AFLP fingerprint pattern for 18 genotypes including four exotics that was derived by using primer combination $E_{ACG} \times M_{CTG}$. In this experiment, a total of 86 amplification products were scored in the 41 neem lines, and the percentage of polymorphism was 69.7%. As can be seen, the four exotics, namely Khao kuang, Ban Huey Sein, Doi Tan and Uthaithani (Fig. 2), were clearly discernible from the rest of the Indian accessions. Of the bands in this experiment 26% were monomorphic across both Indian and the exotic genotypes. Accession-specific bands such as the one marked by the arrowhead in the genotype from Kanpur (third lane from left, Fig. 2) and several rare bands were also identified. The ability to detect polymorphism depends on the primer combination used as evidenced by comparing Figs. 1 and 2.

As observed in Figs. 1 and 2, most of the bands were found in the lower region of the gel, with the number decreasing towards the top. The bands amplified were of uniform intensity across the lanes and did not vary significantly with respect to the initial concentration of the template DNA. Table 3 summarizes the percentage

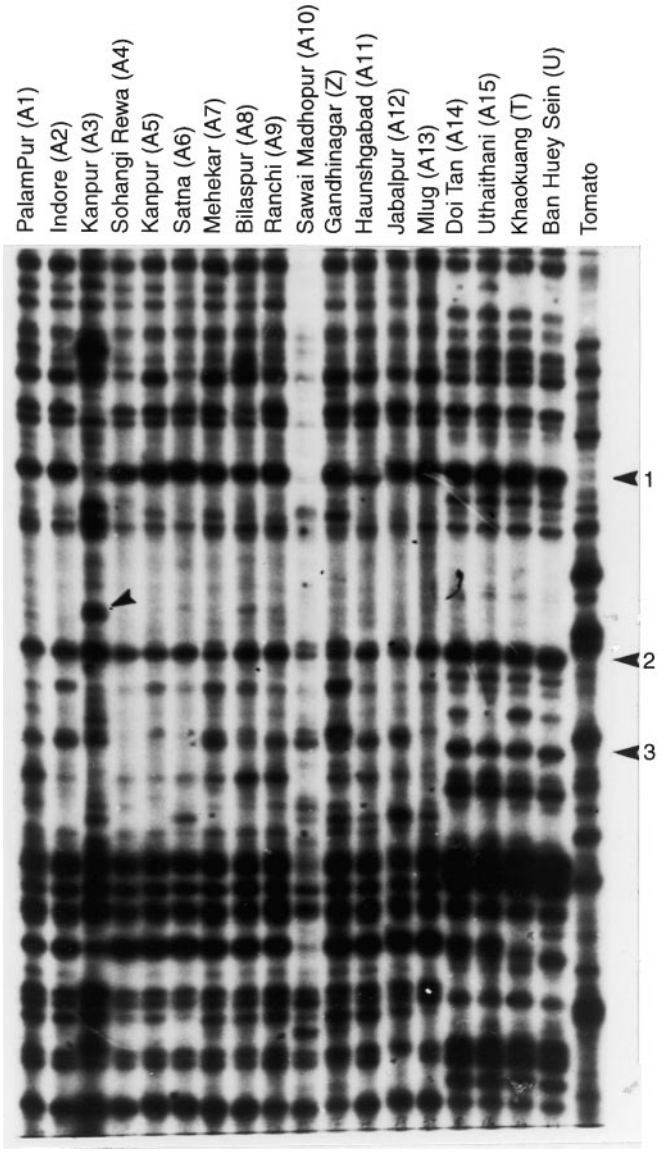

Fig. 2 Autoradiograph displaying AFLP fingerprint detected in 16 neem genotypes using selective primer combination $E_{ACG} \times M_{CTG}$. Lanes marked *T*, *U*, *A14* and *A15* represent exotic varieties from Thailand; the remaining lanes correspond to neem accessions collected from different geographical regions of India (see Table 1). Tomato was used as a control. *Arrowheads 1* and *2* indicate the monomorphic bands, whereas the bands indicated by *arrowhead 3* are present only in Thai genotypes and are thus polymorphic in nature. The band shown in the *Kanpur* (*A3*) lane (*arrowhead*) is genotype-specific

of polymorphism in the neem genotypes using seven different primer combinations. The average number of scorable bands per gel was found to be 60. However, the maximum number of bands was found to be 86 with primer combination $E_{ACG} \times M_{CTG}$, whereas the least number of bands (47) was obtained with primer combination $E_{ACA} \times M_{CAC}$ (Table 3), thus confirming the high multiplex ratio produced by AFLP markers. The highest percentage of polymorphism (83.8%) was obtained with primer combination $E_{ACA} \times M_{CAT}$, while the lowest value, 58%, with primer combination $E_{AAG} \times M_{CTA}$ (Table 3).

Table 3 Information conveyed by seven selective primer combinations employed to detect AFLP among 41 neem genotypes

Primer combination	Total no. of bands in a gel	Percentage polymorphism
$E_{ACA} \times M_{CAT}$	62	83.8%
$E_{AAC} \times M_{CTG}$	54	70.3%
$E_{AAG} \times M_{CTA}$	56	58.0%
$E_{ACA} \times M_{CAC}$	47	68.0%
$E_{ACA} \times M_{CAG}$	59	67.7%
$E_{ACG} \times M_{CAT}$	59	71.1%
$E_{ACG} \times M_{CTG}$	86	69.7%

Genetic similarity matrix and cluster analyses

The AFLP data was used to make pair-wise comparisons of the genotypes based on both shared and unique amplification products to generate a similarity matrix using the NTSYS-pc statistical package (version 1.50, Rohlf 1989). The highest value of similarity coefficient (0.93) was detected between genotypes collected from Mathura and Shivpuri (genotypes M and O, data not shown). Within exotic lines the similarity ranged from 0.92 between Khao kuang and Ban Huey Sein (genotypes T and U, respectively, data not shown) to 0.88 between Ban Huey Sein and Uthaithani (genotypes U and A15, respectively, data not shown).

The similarity matrix representing the Jaccard coefficient was used to cluster the data using the UPGMA algorithm (NTSYS-pc, version 1.5). The resultant dendrogram (Fig. 3) grouped all the Thailand exotics (Khao kuang, Doi Tan, Ban Huey Sein and Uthaithani) in a separate cluster. The rest of the Indian lines were linked to the exotics at a low similarity coefficient of approximately 0.46. Within the Indian cluster, distinct subgroups could be visualized. Subgroup A comprised 16 genotypes belonging to Gurgaon, Pali, Jaisalmer, Mathura, Shivpuri, Nagpur, Ujjain, Kanpur, Indore, Sikar, Nagpur, Bankura, Ravinagar, Haunshgabad, Jabalpur and Mlug. Similarity coefficients for this subgroup ranged from 0.93 (between genotypes from Mathura and Shivpuri) to 0.82 (between genotypes from Nagpur and Bankura). Likewise, subgroup B consisted of 8 lines from Jhansi, Bikaner, Pune, Rajkot, Palampur, Satna, Sohangi Rewa and Mehar. A small subgroup C comprising genotypes collected from Gandhinagar, Kanpur and Bilaspur was also identified. The rest of the genotypes could not be identified as belonging to a discrete cluster.

In order to find out the minimum number of bands that need to be screened to generate a reliable dendrogram depicting genetic similarity, we split the data

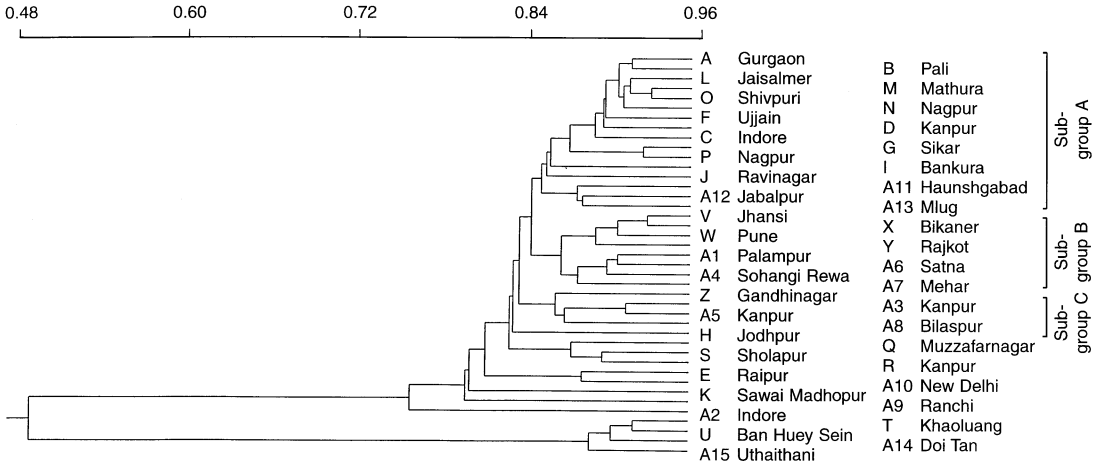

Fig. 3 Dendrogram of 41 neem accessions revealed by UPGMA cluster analysis

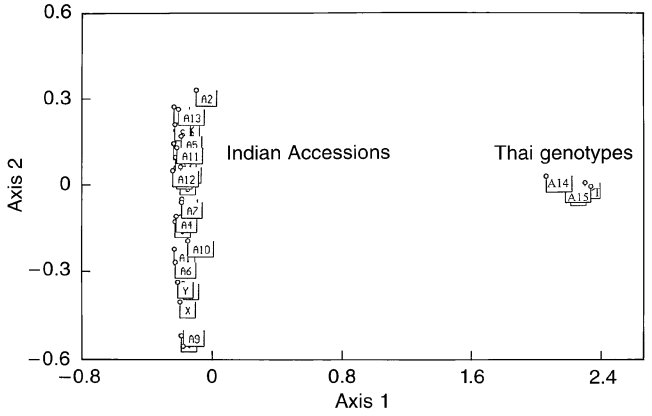

Fig. 4 The plot of 41 neem accessions with 297 polymorphic AFLP markers showing 37 Indian accessions and the four Thailand genotypes present as separate clusters

matrix of 422 bands into two. The first data matrix consisted of the first 203 bands while the second consisted of the remaining bands. The phenetic dendrograms generated from these data sets were compared with the main dendrogram that included all 422 bands. In all three phenetic dendrograms (data not shown) the exotic lines clustered separately. Within the Indian genotypes, the similarity coefficient values remained by and large the same although minor rearrangements were observed. To visualize the genetic relationships among neem accessions in detail, we performed principal correspondence analysis (Fig. 4). The description of data was done using two dimensions. As can be seen in Fig. 4, a clear-cut separation of 37 Indian genotypes from the four Thai accessions was obtained in relation to the first two principal axes of variation. This result is coherent with the dendrogram generated employing UPGMA and is a further confirmation of the genetic similarities delineated by us.

Discussion

With the objective of selecting and maintaining elite trees to bring about an overall genetic improvement in neem for enhanced output of oil, azadirachtin, biomass and adaptibility to stress conditions, provenances of neem have been collected from various regions of the Indian sub-continent and are at present being maintained at AFRI, Jodhpur, India. However, the evaluation and characterization of neem has not been conducted hitherto using molecular markers. The current study was therefore undertaken to document and measure genetic diversity in neem trees growing in North and Central parts of India and parts of Thailand using AFLP markers. The AFLP data generated was highly reproducible with less than 1% error rates as compared to RAPDs (data not shown). Unlike RAPDs, the AFLP profiles did not alter with minor variations in the experimental conditions. This feature may be on account of the high specificity of the AFLP priming reaction conditions that overcomes the usual mismatch annealing associated with RAPD reactions (Neale and Harry 1994). Similar results have been reported in soybean and barley (Becker et al. 1995; Lin et al. 1996) using AFLP methodology. Jones et al. (1997) tested the reproducibility of three popular marker technologies, namely RAPDs, SSRs and AFLPs, and recommended AFLPs as a highly repeatable assay with low error rates. In soybean and barley accessions (Powell et al. 1996; Russell et al. 1997, respectively), AFLPs were reported to display a high marker index as compared to other marker technologies. Thus, AFLP methodology has a potential to screen a large number of genetic loci per experiment (Ellis et al. 1997). These markers can detect more point mutations per hundred nucleotides than RFLP markers. The polymorphism detected by RFLP is mainly due to a variation at the restriction site, whereas with AFLP, an additional number of nucleotides, apart from the restriction site, are screened for polymorphism (Becker et al. 1995). This technique is particularly useful for evaluating genetic diversity in

those plant species where prior information regarding the genome is not available.

The present study revealed that there is a large genetic diversity in neem. On average, 69.8% polymorphism was detected with each AFLP fingerprint, indicating a high marker index. Moreover, no 2 accessions shared a similar DNA profile, indicating that it is possible to uniquely fingerprint all the genotypes. AFLP analysis revealed that the genetic similarity varied from a high of 93% (0.93) to a low of 46% (0.46) within the 41 neem genotypes. 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 in 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 in-breeding types.

Cluster analyses of the neem genotypes employing UPGMA as well as principal correspondence analysis led to the segregation of the accessions into two distinct groups. The 37 Indian genotypes grouped into one cluster, while the four Thai genotypes segregated into a separate cluster. The two clusters were linked at an extremely low similarity coefficient, thereby indicating that the Thai genotypes may actually belong to another species, namely *Azadirachta siamensis*. Similar conclusions were reached by Changtragoon et al. (1994) who, when assessing genetic diversity in *Azadirachta* spp. in Thailand using 11 isozyme systems, identified *A. indica* and *A. indica* var 'siamensis Valenton', as two distinct species. The two varieties of *A. indica* have also been distinguished based on variations in pollen morphology wherein the pollen was found to differ in the thickness of the pollen membrane (Lauridsen et al. 1994). However, a detailed study using molecular markers such as species-specific repetitive DNA sequences and ribosomal DNA intergenic spacers need to be undertaken for confirmation.

Future attempts in neem improvement programs should therefore take into consideration our findings as these have direct implications in tree improvement programs. We have characterized DNA profiles of the selected provenances of 41 neem genotypes using AFLP. Molecular breeding amongst the elite trees, as identified by their respective fingerprints, may be carried out to produce superior clones. Also, if conservation strategies have to be devised to protect neem trees, then populations representing the maximum genetic diversity need to be conserved followed by the population which complements the previous one. Prior to this, however, an exhaustive study with regard to the existing genetic diversity in individual populations of neem needs to be undertaken.

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