View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by AJOL - African Journals Online

academicJournals

Vol. 12(20), pp. 2822-2829, 15 May, 2013
DOI: 10.5897/AJB2013.12352
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic structure and diversity of the neem germplasm bank from Brazil Northeast

Ana Veruska Cruz da Silva[1], Allívia Rouse Carregosa Rabbani[2], Camila Santos Almeida[2] and Débora Clivati[3]

[1]Embrapa Coastal Tablelands. Av. Beira-mar, 3250. Aracaju, Sergipe, 49025-040, Brazil.
[2]Universidade Federal de Sergipe. Laboratory of Genaplant. São Cristovão, Sergipe, 49000-000, Brazil.
[3]Universidade de Mogi das Cruzes. Laboratory of Plant Systematics, Mogi das Cruzes, São Paulo, 08780-911, Brazil.

Accepted 10 May, 2013

Neem (*Azadirachta indica* A. Juss) is a tree species with known value to agriculture. Our aim was to evaluate, using random amplified polymorphic DNA (RAPD) markers, the genetic diversity of 54 accessions from Germplasm Bank (GBN) of Embrapa Coastal Tablelands (Sergipe, Brazil). The accessions were analyzed using a model-based Bayesian procedure (Structure), molecular variance analysis (AMOVA) and Jaccard coefficient was estimated. The marker data indicated that GBN have three independent genetic groups, confirmed by genetic structure and genetic variability, enabling the formulation of appropriate strategies for management and use of GBN.

Key words*: Azadirachta indica, Azadirachta excelsa,* genetic resource, variability.

INTRODUCTION

Azadirachta is a genus of trees belonging to Meliaceae, and present two important species: *Azadirachta excelsa* (Jack) Jacobs, and *Azadirachta indica* A. Juss (Mabberley et al. 1995). Both species, called neem, are native to the Indo-Malaysian region and have been used in agricultural, medical, cosmetic and livestock production (NRC, 1992; Schmutterer, 1995). Neem extracts are being considered an environmentally safe alternative to toxic commercial pesticides (Prakash et al., 2002; Orozco-Sanchez and Rodriguez-Monroy, 2007).

The Northeastern Brazil has ideal conditions for neem development (Neves and Carpanezzi, 2008) and this species has been suggested as an alternative to agriculture. In the municipality of Aracaju, state of Sergipe, is located the Neem Germplasm Bank (GBN), which belongs to Embrapa Coastal Tablelands (Sergipe, Brazil). The GBN characterization is important to identify its accessions and broaden its genetic base, offering diverse materials with high genetic quality. An understanding of the extent and organization of genetic diversity of neem would be valuable for effective assessment, identification, documentation and use of genetic resources for conservation programs and efficient use (Deshwal et al., 2005).

Molecular markers are used to detect genetic variation of genotypes of interest at DNA level. Random amplified polymorphic DNA (RAPD) is one of the tools used, especially in the characterization of genetic resources for the assessment of genetic diversity and relationship measures in various plant species (Degani et al., 2001; Bekessy et al., 2002; Silveira et al., 2009). RAPD

*Corresponding author. E-mail: ana.veruska@embrapa.br; Tel/Fax: (+55) 79 40091362.

Abbreviations: GBN, Neem Germplasm Bank; **RAPD,** random amplified polymorphic DNA; **PCR,** polymerase chain reaction; **PIC,** polymorphic information content; **MI,** marker index; **RPP,** reconstructed panmictic populations; **AFLP,** amplified fragment length polymorphism; **AMOVA,** molecular variance analysis.

Table 1. Neem (*Azadirachta* sp.) accessions analyzed by RAPD markers from Germplasm Bank of Embrapa Coastal Tablelands (Aracaju, Brazil).

Accession	Original location	Accession	Original location
Ae1	Cenargen	Ai28	CPAC
Ae2	Cenargen	Ai29	Cruangi sugar mill
Ae3	Cenargen	Ai30	Cruangi sugar mill
Ae4	Cenargen	Ai31	Cruangi sugar mill
Ae5	Cenargen	Ai32	Cruangi sugar mill
Ae6	Cenargen	Ai33	Cruangi sugar mill
Ae7	Cenargen	Ai34	Cruangi sugar mill
Ae8	Cenargen	Ai35	Cruangi sugar mill
Ae9	Cenargen	Ai36	Cruangi sugar mill
Ae10	Cenargen	Ai37	Cruangi sugar mill
Ae11	Cenargen	Ai38	Cruangi sugar mill
Ae12	Cenargen	Ai39	Cruangi sugar mill
Ae13	Cenargen	Ai40	Cruangi sugar mill
Ae14	Cenargen	Ai41	Cruangi sugar mill
Ae15	Cenargen	Ai42	Petrobras
Ae16	Cernargen	Ai43	Petrobras
Ae17	Cenargen	Ai44	Petrobras
Ae18	Cenargen	Ai45	Petrobras
Ae19	Cenargen	Ai46	Petrobras
Ae20	Cenargen	Ai47	Petrobras
Ae21	Cenargen	Ai48	Petrobras
Ae22	Cenargen	Ai49	Petrobras
Ai23	CPAC	Ai50	Petrobras
Ai24	CPAC	Ai51	Petrobras
Ai25	CPAC	Ai52	Petrobras
Ai26	CPAC	Ai53	Petrobras
Ai27	CPAC	Ai54	Petrobras

markers are mostly dominant and detect variation; they are technically simple, suitable for large-scale germplasm characterization and can be performed even in a moderately equipped laboratory (Deshwal et al., 2005; Costa et al., 2011).

In view of the importance of neem in the Northeastern Brazil, our study aims to evaluate the genetic structure and genetic relationships among the 54 accessions from Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil), using RAPD markers.

MATERIALS AND METHODS

Overall, there are 54 accessions in the GBN: 22 of *A. excelsa*, donated by Cenargen - Embrapa Genetic Resources and Biotechnology (Brasília, Brazil); and 32 of *A. indica*, donated by CPAC - Embrapa Cerrados (Brasília, Brazil), by Cruangi sugar mill (Pernambuco, Brazil), and collected in reforestation area of Petrobras (Sergipe, Brazil) (Table 1). We studied the 54 accessions from GBN located in the Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

We used young fresh leaves for DNA extraction following the method of Doyle and Doyle (1987). Each RAPD reaction was performed in 25 µL volume containing 50 ng genomic DNA, 1X PCR buffer (Gibco-BRL, Grand Island, NY, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 U Taq DNA polymerase (Gibco, Grand Island, NY, USA), 30 ng/µL primer and 20 µL ultrapure water.

The polymerase chain reaction (PCR) amplifications were performed using a PTC-100 thermocycler (Programmable Thermal Controller - MJ Research, Inc.) and subjected to a cycle of 96°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 96°C for 45 s, 36°C for 45 s for primer annealing, 72°C for 45 s for extension, and finally one cycle of 72°C for 5 min for final extension (Silva et al., 2012). We tested 10 RAPD primers (A10, A11, A13, A15, A19, A2, B11, K20, W13 and IDT14) with nine Operon primers (Operon Technologies, USA) and one IDT primer (Integrated DNA Technologies, Germany) (Table 1).

For electrophoresis, 10 µL of PCR products were mixed to 1.5 µL of blue juice (0.01% bromophenol blue, 40% glycerol). We used 1.5% agarose gel (1X TEB - 89 mM TRIS, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system Sunrise (Gibco BRL, Grand Island, NY, USA), carried out at a constant voltage of 100 V for 90 min. Gel was stained with ethidium bromide solution (5 mg/mL) for 15 min, and the amplification products were visualized under ultraviolet light using a Gel Doc L-Pix image system (Loccus Biotecnologia, Brazil).

The electrophoretic profile of each gel was transformed into a

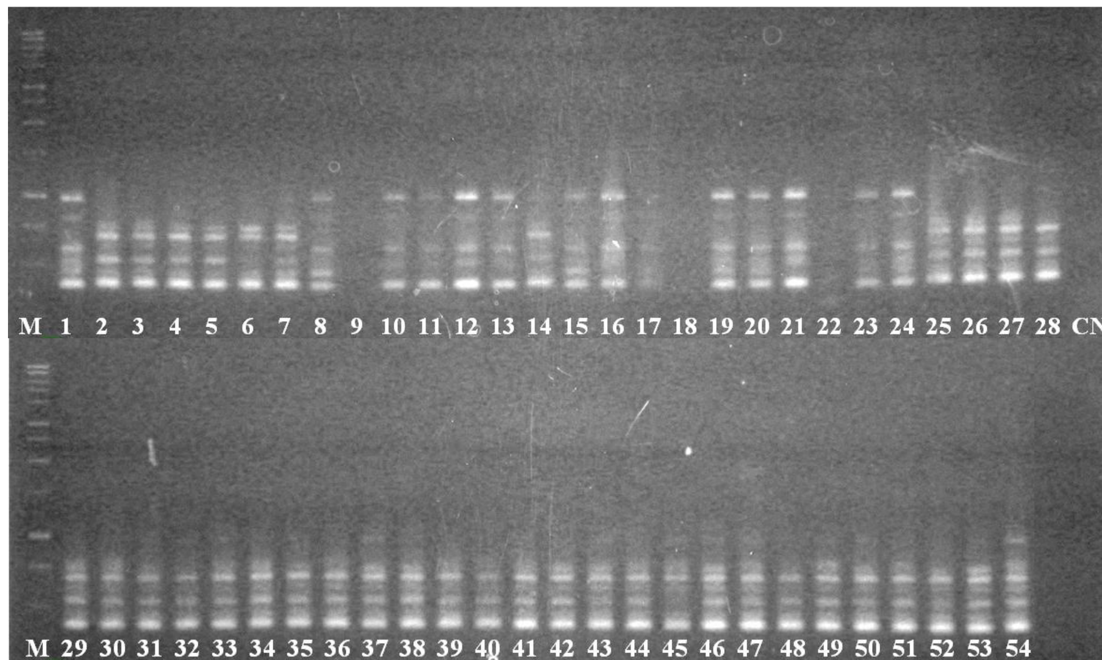

Figure 1. RAPD profiles generated by A13 primer among 54 neem accessions (*Azadirachta* sp.), with marker (M) and negative controls (CN). Accessions from Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

binary matrix of presence (1) and absence (0), which was subsequently used for all analysis. Bootstrap procedure was applied to calculate variance of the genetic distance obtained from markers, and was obtained from 5.000 bootstrap random draws using the DBOOT software (Coelho, 2001). Polymorphic information content (PIC) for dominant marker was calculated as suggested by Ghislain et al. (1999):

$$PIC = 1 - p^2 - q^2$$

Where, p is band frequency and q is no band frequency. The marker index (MI) was determined as a product of PIC and the number of polymorphic bands per assay unit as described by Zhao et al. (2007). To measure the genetic diversity, we used Genalex v.6.3 (www.anu.edu.au/BoZo/GenAlEx/) and calculated the Shannon Index (I) (Brown and Weir, 1983 and the genetic diversity (H) as described by Lynch and Milligan (1994) and Maguire et al. (2002) for dominant markers.

RAPD markers were scored for the presence (1) or absence (0) of a fragment, and a data matrix of I-scores were generated and similarity coefficients calculated using Jaccard's arithmetic complement index Jaccard (1908). The dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) cluster algorithm (Sokal and Michener, 1958), in order to determine the robustness of the dendrogram; the data was bootstrapped with 10.000 replications using FreeTree software (http://web.natur.cuni.cz/flegr/programs/freetree.htm). For cluster visualization we used the TreeView package (http://web.natur.cuni.cz/flegr/programs/freetree/TreeView.exe).

Inference about the genetic structure within neem was obtained using the software STRUCTURE version 2.2 (Pritchard et al., 2000; Falush et al., 2007). For our analysis, each class of genotypes was treated as containing haploid alleles, following the software protocol and Oliveira et al. (2010). We estimated the number of K (unknown) reconstructed panmictic populations (RPPs) of individuals, using values ranging from 1 to 10, assuming that the sampled genotypes were from anonymous plants of unknown origin (we used the options usepopinfo = 0, popflag = 0). We set up runs with a burn-in period of 30,000 and a Monte Carlo Markov chain (MCMC) of 500.000, with five repetitions. The program Structure estimates the most likely number of clusters (K) by calculating the log probability of data for each value of K (Dos Santos et al. 2011). We assessed the best K-value supported by the data according to Evanno et al. (2005).

RESULTS

The primers used resulted in a banding pattern that was distinct (Figure 1), which shows the RAPD profiles of the primer A13. The 17 primers generated a total of 43 fragments; 100% polymorphic. Primers with highest number of fragments (eight) were A13 and B12 (Table 2). The primer A15, A2 and W13 had the lowest number of fragments (two). The PIC value was 0.33 (0.25 to 0.43), and the global value MI was 1.37 (0.58 to 2.74). There is a directly proportional relationship between the number of fragments analyzed and the variance (Figure 2). The results indicate a clear variance decrease while the number of fragments increased.

A Bayesian analysis (structure) was conducted using 43 fragments to determine the genetic structure among the neem accessions. The analysis produced a maximum probability for K = 2 (Figure 3A). However, as indicated

Table 2. Primers, totals (TF), polymorphic fragments (PF%), polymorphic information content (PIC), marker index (MI), Shannon index (I) and genetic diversity (H) among 54 neem (*Azadirachta* sp.) accessions from Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

Primer	Sequence 5' - 3'	TF	PF (%)	PIC	MI
A10	GTG ATC GCA G	5	100	0.25	1.25
A11	CAA TCG CCG T	5	100	0.29	1.46
A13	CAG CAC CCA C	8	100	0.34	2.74
A15	TTC CGA ACC C	2	100	0.39	0.79
A19	CAA ACG TCG G	5	100	0.26	1.29
A2	TGC CGA GCT G	2	100	0.39	0.79
B11	GTA GAC CCG T	8	100	0.29	2.30
IDT14	GGC ACT GAG G	3	100	0.43	1.30
K20	GTG TCG CGA G	3	100	0.41	1.22
W13	CAC AGC GAC A	2	100	0.29	0.58
Total		43	100	0.33	1.37

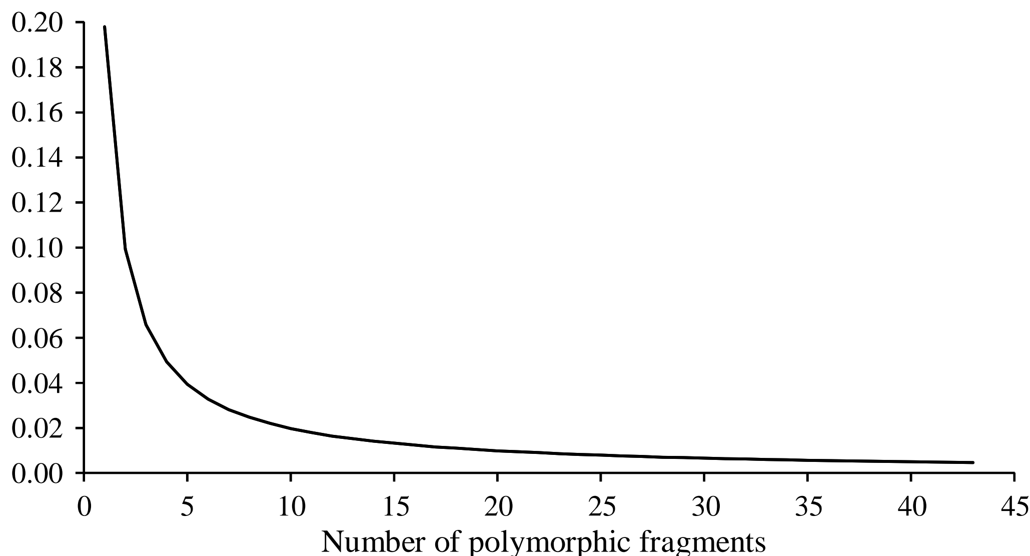

Figure 2. Variance of the polymorphic fragments using RAPD markers between 54 neem (*Azadirachta* sp.) accessions belonging Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

by Evanno et al. (2005), and subsequently shown in sorghum (Barnaud et al., 2007), chestnut (Pereira-Lorenzo et al., 2011) and pear (dos Santos et al. 2011), other submaximal can indicate the presence of sub-structure. In this study, the K = 3 was the second sub-maximal. When analyzing the data for K = 3 (Figure 3B), reconstructed populations were distinguished for two groups of *A. indica*, and a third group of *A. excelsa*.

We assumed three clusters the substructure of GBN (Figure 3B). The first Reconstructed Populations (RPP1) grouped 14 accessions of *A. indica*; 12 of them had probability of membership (q*I*) > 80%. The second RRP (RPP2) assigned 24 genotypes, 20 of them had a q*I* > 80%, including seven genotypes identified as *A. excelsa* (Ae14, Ae2, Ae3, Ae4, Ae5, Ae6, Ae7). The third RRP (RPP3) clustered 16 genotypes, 14 of which had a q*I* > 80%, including one genotype initially identified as *A. indica* (Ai23). The genotypes identified as *A. excelsa* that grouped with *A. indica* also showed the same characteristic for K = 2 (RPP1) with q*I* > 80%. The genotype Ai23 (RPP2, q*I* > 80%) was identified as *A. excelsa* (Figure 3A).

The genetic distances between the neem accessions, as estimated by Jaccard similarity coefficients (JC), were used to build a UPGMA dendrogram (Figure 4), after bootstrapping the data 10000 times. To 54 genotypes, the similarity mean found was 0.45 (0.00 to 0.95). Within the G2 group, we observed the formation of two sub-

Figure 3. The reconstructed populations, K=2 (A, RPP1 to 3) and K=3 (B, RPP1 to 3) defined using Structure (Pritchard et al. 2000) for 54 neem (*Azadirachta* sp.) accessions belonging Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

groups according to the genotypes origins. SG1 for the group of genotypes originating from Petrobras (Sergipe, Brazil) at 0.68 JC and SG2 for the genotypes assigned by Cruangi sugar mill (Pernambuco, Brazil) at 0.59 JC. Also, within the group *A. indica,* the genotype Ai42 diverged at a 0.57 JC level, been isolated when compared with other accessions.

All accessions differed from each other. The genotype Ae9 differed at 0.00 level of similarity with other 14 genotypes (Ae22, Ai29, Ai30, Ai31, Ai32, Ai33, Ai37, Ai38, Ai39, Ai40, Ai42, and Ai43 Ai49), another three pairs also have the same characteristics (Ae18, Ae30; Ae18, Ai33; Ae22, Ae9). We found four pairs (Ae5, Ae4; Ae6, Ae4; Ai46, Ai52; Ai48, Ai51) presenting the greatest similarity within the collection, with 0.95 JC.

UPGMA, such as detected by Bayesian analysis, identified some genotypes classified as *A. excelsa* mixed with *A. indica*, and vice versa. This shows an error of identification in GBN, based only on morphological characteristics. For example, seven accessions identified as *A. excelsa* (Ae14, Ae3, Ae2, Ae7, Ae6, Ae4 and Ae5) are grouped in the G2, the *A. indica* group. The same way, two genotypes (Ai24 and Ai23) identified primarily as *A. indica* were found in the G3, the *A. excelsa* group. The genotype Ae22 was the most different from GBN, isolated in a branch with 0.19 JC. Other three genotypes (Ae9, Ae18 and Ae17) also differed, grouping together with 0.13 JC. All accessions belong to the species *A. excelsa.* These four accessions were in the first population reconstructed (RPP1) all with a q*I* > 80%. The RPP3 defined by the Structure software, corresponded to the G2 of UPGMA Cluster, and the most genotypes present in the G3 group corresponded to RPP2.

AMOVA was performed among the 54 different accessions, grouped by their original location. The accessions showed low genetic differentiation (32%). When analyzed by RPPs groups, the genetic differentiation only accounted for 61% of the variation (Table 3). For the genetic structure, the Shannon's Index (I) found was 0.37 and to genetic diversity (H) the value estimated was 0.25 to RPPs (Table 4).

DISCUSSION

The results indicate a clear decrease of variance while the number of fragments increases. According to Moura et al. (2005) and Costa et al. (2011), there is a point where the increase of the number of fragments does not show a significant increase in experimental accuracy, which does not justify an extra effort in labor (Bekessy et al., 2002). From 35 fragments on, the variance stabilizes with value less than 0.01, suggesting that the results obtained by the fragments used in this study (43) can be used for diversity analysis.

The RAPD markers were used in many parts of the world to measure genetic fidelity and the genetic diversity

Figure 4. Dendrogram of genetic similarity generated using the Jaccard coefficient and the UPGMA method with analysis bootstraps for 54 neem accessions (*Azadirachta* sp.) belonging to Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil).

Table 3. Analysis of molecular variance (AMOVA) for the 54 neem (Azadirachta sp.) accessions belonging to Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil) having as source of variation the original location and reconstructed populations (RPPs) defined by the structure and dendrogram groups.

Original location	df	Estimated variance	Variation (%)
Among	3	2.58	32%***
Within	50	5.57	68%
RPPs (K=3)			
Among	2	4.68	52%***
Within	51	4.33	48%
Dendrogram Group			
Among	2	6.81	62%***
Within	50	4.22	38%

df, Degrees of freedom; ***P<0.001.

Table 4. Values of Shannon's Information Index (I) and genetic diversity (H) to neem (Azadirachta sp.) accessions belonging to Embrapa Coastal Tablelands (Aracaju, Sergipe, Brazil) by original location, reconstructed populations (RPPs) defined by the Structure software (Pritchard et al. 2000) and dendrogram groups.

Original location	I	H
Cenargen	0.46	0.30
CPAC	0.34	0.23
Cruangi Factory	0.26	0.17
Petrobras	0.26	0.18
Mean	0.33	0.22
RPPs (K=3)		
RPP1	0.27	0.18
RPP2	0.31	0.20
RPP3	0.37	0.25
Mean	0.32	0.21
Dendrogram Group		
G2	0.35	0.23
G3	0.25	0.16
G4	0.05	0.04
Mean	0.22	0.14

in species of neem (Farooqui et al., 1998; Dhillon et al., 2007; Arora et al., 2010). The high level of polymorphism observed in this study agrees with results of previous genotyping studies using RAPDs (Deshwal et al., 2005). However, the polymorphism level produced by RAPD markers in our study was higher than in other case, possibly due to the better representativeness of neem diversity in the Germplasm Bank from Sergipe (Brazil).

Genetic diversity indicated by I and H in GBN can be considered low. The mean values were less than 0.50, as explained by Costa et al. (2011) who analyzed accessions of Mangaba Germplasm Bank from Sergipe (Brazil). On account of low diversity in GBN, it is necessary to insert a new access to promote increased diversity and better use these genetics resources. In our study, we found a wide range of genetic similarity among accessions (0.00 to 0.95), more than Deshwal et al. (2005) who reported a similar genetic (0.70 to 0.96) relationship among 29 neem accessions collected from two agro-ecological regions of India, which cover three states, Punjab, Haryana and Rajasthan using RAPD markers; and Singh et al. (1999) to 37 neem accessions (0.74 to 0.93) from different eco-geographic regions of India and four exotic lines from Thailand using amplified fragment length polymorphism (AFLP) markers. Allogamous woody plants usually display considerable variability (Hamrick, 1990), which is true in the present study.

The structure analysis and cluster analysis has clearly indicated that there is low isolation among the accessions collected from origins, revealing a high variability in three RPPs. This higher variability should be due to the presence of more than one species within the collection, as confirmed by AMOVA. These are powerful tools for detecting the structure in a germplasm bank, as already demonstrated by other authors (dos Santos et al., 2011; Pereira-Lorenzo et al., 2011).

This study was the first to use RAPD markers to assess genetic diversity in neem accessions in northeastern Brazil. None of the individual plants were genetically identical according to the Jaccard similarity index, which indicated that the level of resolution in this study was sufficient to distinguish all genotypes. According to the UPGMA dendrogram based on the Jaccard similarity index, in general the genotypes were grouped by origin and specie, as also confirmed by bootstraps and Bayesian analysis.

Our results may help the GBN restructuring, with the correct classification of genotypes, because it allows inferences about their current status and proposes measures to either maintain the conservation condition or to recover the genetic potential of the species, as noted by Zimback et al. (2004) and Bertoni et al. (2010). Allying this, future studies should be developed with respect to features of interest in the GBN accessions. The genetic information associated with the agronomic characteristics can be used in future breeding work and selection of better genotypes.

Conclusions

This is the first report on neem species in northeastern Brazil, and our study is a contribution to the characterization and genetic fidelity of accessions. The genetic variation and genetic relationships among neem accessions were efficiently determined using RAPD markers. The GBN shows three independent genetic groups, confirmed by genetic structure and genetic variability, enabling the formulation of appropriate strategies for conservation and improvement programs.

REFERENCES

Arora K, Sharma M, Srivastava J, Ranade SA, Sharma AK (2010). Rapid in vitro cloning of a 40-year-old tree of *Azadirachta indica* A. Juss. (Neem) employing nodal stem segments. Agrof. Sys. 78:53-63.

Barnaud A, Deu M, Garine E, McKey D, Joly HI (2007). Local genetic diversity of sorghum in a village in northern Cameroon: structure and dynamics of landraces. Theor. Appl. Genet. 144:237-348.

Bekessy SA, Allnutt TR, Premoli AC, Lara A, Ennos RA, Burgman MA, Cortes M, Newton AC (2002). Genetic variation in the vulnerable and endemic monkey puzzle tree, detected using RAPDs. Heredity 88:243-249.

Bertoni BW, Souza AV, Biondo R, França SC, Telles MPC, Pereira MAS (2010). Genetic diversity among natural populations of *Mandevilla velutina*. Hortic. Bras. 28:209-213.

Brown AHD, Weir BS (1983). Measuring genetic variability in plant populations. In: Tanksley SD, Orton TJ (eds) Isozymes in Plant Genetics and Breeding, Elsevier, Amsterdam. pp. 219-239.

Coelho SG (2001). Software DBOOT: Evaluation of errors associated with estimates of genetic distances/similarities by bootstrap procedure with variable markers versão 1.1. Universidade Federal de Goiás, Goiânia, Brazil.

Costa TS, Silva AVC, Lédo AS, Santos ARF, Silva Junior JF (2011). Diversidade genética de acessos do banco de germoplasma de mangaba em Sergipe. Pesq. Agrop. Bras. 46:499-508.

Degani C, Rowland LJ, Saunders JA, Hokanson SC, Ogden E L, Golan-Goldhrish A, Galletta GJ (2001). A comparison of genetic relationship measures in strawberry (*Fragaria ananassa* Duch.) based on AFLP, RAPDs, and pedigree data. Euphytica. 117:1-12.

Deshwal RPS, Singh R, Malik K; Randhawa GJ (2005). Assessment of genetic diversity and genetic relationships among 29 populations of *Azadirachta indica* A. Juss. using RAPD markers. Genet. Resour. Crop. Evol. 52:285-292.

Dhillon RS, Mohapatra T, Singh S, Boora KS, Singh, K (2007). Assessment of genetic diversity in *Azadirachta indica* based on DNA fingerprinting. Indian J. Biotech. 6:519-524.

Dos Santos ARF, Ramos-Cabrer AM, Diaz-Hernandez M, Pereira-Lorenzo S (2011). Genetic variability and diversification process in local pear cultivars from northwestern Spain using microsatellites. Tree Gen. Genome 7:1041-1056.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11‑15.

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611-2620.

Falush D, Stephens M, Pritchard JK (2007). Inference of population structure using multilocus genotype data: Dominant markers and null alleles. Mol. Ecol. Notes. 7:574-578.

Farooqui N, Ranade SA, Sane PV (1998). RAPD profile variation amongst provenances of neem. Bioch. Mol. Bio. Int. 45: 931-939.

Ghislain M, Zhang D, Fajardo D, Huamann Z, Hijmans RH (1999). Marker assisted sampling of the cultivated Andean patato *Solanum phureja* collections using RAPD markers. Gen. Res. Crop Evol. 6:547-555.

Hamrick JL (1990). Isozyme and the analysis of genetic structure in plant populations. In: Soltis ED, Soltis PS (eds) Isozyme in Plant Biology, Chapman and Hall, London. pp. 87-105.

Jaccard P (1908). Nouvelles recherches sur la distribution florale. Soc. Vaud. Sci. Nat. 44:223-270.

Lynch M, Milligan BG (1994). Analysis of population genetic structure with RAPD markers. Mol. Ecol. 3: 91-99.

Mabberley DJ, Pannell CM, Sing AM (1995). Meliaceae - Flora Malesiana series I, Vol. 12(1). Foundation Flora Malesiana, Leiden, Netherlands.

Maguire TL, Peakall R, Saenger P (2002). Comparative analysis of genetic diversity in the mangrove species *Avicennia marina* (Forsk.)Vierh. (Avicenniaceae) detected by AFLPs and SSRs. Theor. Appl. Gen. 104:388-398.

Moura NF, Chaves LJ, Vencovsky R, Zucchi MI, Pinheiro JB, Morais LK, Moura MF (2005). Selection of RAPD markers to study genetic structure of *Hancornia speciosa* Gomez. Biosci. J. 21:119‑125.

Neves EJM, Carpanezzi AA (2008). O cultivo do nim para produção de frutos no Brasil. Circular Técnica n. 162, Colombo, Brazil.

NRC - National Research Council (1992). Neem: A Tree for Solving Global Problems. National Academy Press, Washington, USA. P.152.

Oliveira LO, Venturini BA, Rossi AAB, Hastenreiter SS (2010). Clonal diversity and conservation genetics of the medicinal plant *Carapichea ipecacuanha* (Rubiaceae). Gen. Mol. Bio. 33:86-93.

Orozco-Sanchez F, Rodriguez-Monroy M (2007). Cell suspension culture of *Azadirachta indica* for production of a bioinsetide. Rev. Mex. Ing. Quí. 6:251-258.

Pereira-Lorenzo S, Lourenço CR, Ramos-Cabrer AM, Ciordia-Ara M, Marques RC, Borges O, Barreneche T (2011). Chestnut cultivar diversification process in the Iberian Peninsula, Canary Islands and Azores. Genome 54:301-315.

Prakash G, Bhojwani S, Srivastava A (2002). Production of azadirachtin from plant tissue culture: state of the art and future prospects. Biotechnol. Bioprocess Eng. 7:185-193.

Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Schmutterer H (1995). The neem tree *Azadirachta indica* A. Juss. and other malicious Plant: source of unique natural products for integrated pest management, medicine, industry, and other purposes. Weinheim, Federal Republic of Germany, Germany.

Silva AVC, Santos ARF, Lédo AS, Feitosa RB, Almeida CS (2012). Moringa genetic diversity from germplasm bank using RAPD Markers. Trop. Subtrop. Agroec. 15:31-39.

Silveira DG, Amorim EP, Jesus ON, Souza FVD, Pestana KN, Santos VJ, Santana JRF (2009). Genetic variability estimated among caroá populations through RAPD markers. Pesq. Agrop. Bras. 44: 283-290.

Singh A, Negi MS, Rajagopal J, Bhatia S, Tomar UK, Srivastava PS, Lakshmikumaran M (1999). Assessment of genetic diversity in *Azadirachta indica* using AFLP markers. Theor. Appl. Genet. 99:272-279.

Sokal RR, Michener CDA (1958). Statistical method for evaluating systematic relationships. Sci. Bull. 38:1409-1738.

Zhao K, Zhou M, Chen L, Zhang D, Robert GW (2007). Genetic diversity and discrimination of *Chimonanthu spraecox* (L.) link germplasm using ISSR and RAPD Markers. Hortscience 42:1144-1148.

Zimback L, Mori ES, Kageyama PY, Veiga RFA, Mello Junior JRSM (2004). Genetic structure of *Trichillia pallida* Swarts (Meliaceae) populations by RAPD markers. Sci. For. 65:114-119.