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IN VIVO AND *IN VITRO* RAPD ANALYSIS IN APOCYNACEAE FAMILY: POPULATION GROWING IN DIFFERENT REGIONS OF RAJASTHAN, INDIA

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ABSTRACT
The present investigation included five samples hence, PCR-based DNA fingerprinting namely RAPD analysis was done to establish the relationship between the selected species of Rajasthan, India and *in vivo* and *in vitro* comparison of the same species. The RAPD analysis was conducted after isolation and purification the DNA of the all sample. In the present studies, the *in vivo* accessions of both plant samples 1, 2, 3, 5 and 2, 3, 4, 5 were collected from different geographical locations of Rajasthan, India which tend to accumulate genetic variations during the course of environmental adaptations. This Genetic diversity is also of great importance to the sustainability of plant population as there is a possibility of free or random pollen flow and fertilization. The identification of desert plants is crucial for their preservation and sustainable use, as well as to prevent forgery in the marketing of medicinal plants. Expected chemicals in the herbal plant targeted for medicinal use could vary with the genomic or environmental variability of the species.
Keywords: *Nerium oleander, Thevetia peruviana,* PCR, DNA fingerprinting, RAPD.

INTRODUCTION

India is a treasure to a large reservoir of biological wealth. Access to a broad genome enables us to cope up with the challenges for food production that arise from several biotic and abiotic stresses. Cross breeding of these varieties is only possible if a wide range of genes are available within them. Despite our crucial dependence on it, the threat to bio-wealth is increasing every day (Ivy *et al.,* 2010; Manohar and Murthy, 2011). The RAPD technique has several advantages such as ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William *et al.,* 1990). Molecular marker can provide a spectacular improvement in the efficiency and sophistication of plant breeding selection of a correct marker system depends upon the type of study to be undertaken and whether that marker system would fulfill at least a few of the above mentioned characteristics such as easy availability, highly polymorphic nature, Mendelian inheritance, frequent occurrence in genome, selective neutral behavior, easy and fast assay, high reproducibility, free of epistasis, pleiotropy etc (Weising *et al.*, 1995). Molecular genetics techniques using DNA polymorphism is increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (Neill *et al.,* 2003). Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers (Ahmad *et al.,* 2010). The evaluation of the genetic diversity would promote the efficient use of genetic variation in the breeding program (Paterson *et al.,* 1991). Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led to the development of several novel genetic assays based on selective amplification of DNA (Erlich, 1989). In contrast, molecular markers, based on DNA sequence polymorphisms are independent on environmental conditions and show higher levels of polymorphism.

MATERIAL AND METHODS

The experimental materials of both the plants i.e. *Nerium oleander* L. and *Thevetia peruviana* (Pers.) Schum. were collected from the *in vivo* grown plants and callus from nursery of University of Rajasthan, Jaipur, India from Chambal garden Kota, Agriculture University, Bikaner and nursery of Phulera and authenticated by Herbarium, Department of Botany, University of Rajasthan, Jaipur, India. *In vitro* maintained stock callus cultures of *Nerium oleander* and *Thevetia Peruviana* callus was developed from leaf explants on MS medium supplemented with 2,4-D + BAP (4.0 mg/l + 3.0 mg/l) and 2,4-D + Kn (2.0 mg/l +2.0 mg/l) in *Nerium oleander* and *Thevetia peruviana,* respectively.

Reagents

During the present research work, DNA isolation was done by using the method by Doyle and Doyle (1990), which was later modified by Sharma *et al.,* 2003. For DNA isolation 3.0 g of leaves and *in vitro* callus were crushed in mortar and pestle in solution containing 5 ml/g absolute alcohol, chloroform (70:30). After that alcohol 0.5 M EDTA, pH-8 (70:30) were mixed and kept for 30 minutes to denature enzymes. The homogenized material was transferred to 0.75 ml pre-warmed (60 C) DNA isolation buffer (2 CTAB DNA Extraction buffer:- 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2 % CTAB and 2 l/ml -mercaptoethanol) in capped polypropylene tubes.

- The clump was suspended by using spatula.
- Incubated for 1 hour at 60ºC with occasional mixing by gentle swirling in water bath.
- After removing from water bath one volume of chloroform: Isoamyl alcohol (24: 1) was added and mixed by inversion for 15 minute to ensure emulsification of phases.
- Centrifuged at 15000 RPM for 10 minutes.
- Step 4 and 5 was repeated.
- Aqueous phase was taken and transferred to another tube.
- Ice-cold 0.6 ml volume of iso propanol was added to precipitate DNA.

- DNA-CTAB complex was precipitated as a fibrous network; amorphous precipitation was collected by the centrifugation at 15000 RPM for 15 minutes at 20ºC.
- 0.5 ml of 70 % alcohol was added to pellet and was kept for 20 minutes with gentle agitation.
- The pellet was collected by centrifugation at 10000 RPM for 5 minutes at 20ºC.
- The tube was inverted and drained on a paper towel. The pellet was dried over-night after covering with Para film with tiny pores.
- The pellet was re-dissolved in 100 µl of $T_{10}E_1$-buffer by keeping overnight at 40ºC without agitation.

Purification of DNA

Crude extract of plant leaves and callus for DNA isolation was made by the method described by Sharma *et al.* (2003), crude extract of plant have a mixture of DNA, RNA and protein. Hence, to get pure DNA, RNA was removed by treating the sample with DNase free RNase. Protein including RNase was removed by treating with chloroform: Isoamylalcohol (24:1). The purification was carried out in following steps-

- 2.5 µl of RNase was added to 0.5 ml of crude DNA preparation (2.5 µl of RNase = 25 µg of RNase, so treatment was 50 g/ml of DNA preparation).
- It was gently mixed and was incubated at 37º C for 1 hour.
- After 1 hour, a mixture of 0.3 – 0.4 ml of chloroform: Isoamyl alcohol (24: 1) was added and mixed thoroughly for 15 minutes till an emulsion was formed.
- Centrifuged for 15 minutes at 15000 RPM.
- Supernatant was taken avoiding the whitish layer at interface.
- The DNA was re-precipitated by adding double quantity of absolute alcohol.
- To pellet the DNA the tube was centrifuged for 5 minutes at 5000-10000 RPM for 10 minutes.
- The transparent and viscous pellet was washed with 70 percent alcohol and dried over night.
- The DNA was re-dissolved in 200 µl of $T_{10}E_1$ buffer.

Gel analysis

The integrity of DNA was judged through gel analysis in following steps-

- Casted agarose gel (0.8 %) 150 ml in 0.5 X TBE (Tris borate EDTA) buffer containing (0.5 g/ml) of ethidium bromide.
- 5 µl of DNA sample was loaded with a mixture of DNA loading dye.
- Loaded a known amount of uncut λ phage DNA as control in the adjacent well.
- The gel was electrophoresis at 50 V for 1.5 hours.
- Visualized gel under UV light.
- Presence of single compact band at the corresponding position to phase DNA indicated high molecular weight of isolated DNA.

Quantitation of DNA

The quantization of DNA was done by observing it at 260 nm and 280 nm wavelengths by using a spectrophotometer (Nano Drop ND-1000 version V3.1.1) in following steps-

- 2 µl of double distilled water was loaded in the lower pedestal to initialize the spectrophotometer.
- The wavelength was fixed at 260 and 280 nm.
- The 2 µl of $T_{10}E_1$ was loaded for blank measurement.
- The quantities of unknown samples were recorded as ng/l of genomic DNA.

Dilution of DNA for PCR

The quantified DNA was diluted to final concentration of 25 mg/l in $T_{10}E_1$, buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

RAPD analysis

For RAPD analysis 25 primers from set # 1 obtained from the genuine chemical Corporation University of British Columbia, Vancouver, Canada was screened. Out of 25 primers, 10 primers were amplified and selected for the study; rest 15 primers were removed because of their monomorphic nature and poor reproducibility. The PCR reactions were performed in a 25 µl reaction mixture containing:

Distilled water 14.5 µl
Taq Buffer 2.5 µl (10X Assay Buffer (Bangalore Genei)
dNTPs 2.0 µl (200 µM each of dNTPs (Fermentas)
Taq pol. 0.5 U (Bangalore Genei)
Primer 4.0 µl (UBC Set # 2)
Template 2.0 µl

The PCR reactions were carried out in DNA thermal cycler (Model-CGI-96, Corbett Research, Australia) using a single primer in each reaction following the cycling parameter:

Step 1:
Initial denaturation (94ºC) for 4 Minutes

Step 2: (Cycle 1 – 44)
Denaturation (94ºC) 1 Minute
Primer annealing (37ºC) 1 Minute
Primer Extension (72ºC) 2 Minutes

Step 3:
Final extension (72ºC) 7 Minute

Following the amplification, the PCR products were loaded on 1.2 % Agarose gel (Himedia, molecular grade), which was prepared in 1X TBE buffer containing 0.5 µg/ml of the Ethidium Bromide. The amplified products were electrophoresis for 3 - 3.5 h at 100 V with cooling. After separation the gel was viewed under UV trans-illuminator and photographed by digital camera.

Similarity Matrix and Cluster Analysis

The statistical calculations were done using Free Tree programme version, 0.9.1.50 (Pavlicek *et al.* 1999). Similarity matrix for both RAPD primers was constructed using the Jaccard's similarity coefficient values to find out genotypic relationship. The average distance of a single variety from rest of the genotypes was also calculated. The 0/1 matrix data obtained from RAPD primers was arranged to get separate similarity matrix which was subjected to UPGMA (unweighted pair-group method with arithmetic averages) analysis to generate dendrogram. The equation for calculating Jaccard's similarity coefficients 'F' between two samples A and B is:

$$f = n_{x\,y} / (n_l - n_z)$$

n_{xy} = Number of bands common to sample A and sample B.
n_l = Total number of bands present in all samples.
n_z = Number of bands not present in sample A or B but found in other samples.

Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Paired Group Method with Arithmetic Mean) clustering method. The genetic distance obtained from cluster analysis through UPGMA was used to construct the dendrogram, depicting the relationships of the clones using computer program NTSYS pc version 2.02 (Rohlf, 1998). The dendrogram showing relationship among various genotypes was constructed using these clusters.

DNA Isolation, Purification and RAPD Analysis

Random amplified polymorphic DNA (RAPD) analysis was performed with 10 decamer primers purchased from Genuine Chemical Corporation, Bangalore. 10 primers generated in both the plants species (GCC 23, GCC 151, GCC 104, GCC 121, GCC 51, GCC 112, GCC 103, GCC 135, GCC 184, GCC 61 in *Nerium oleander* and GCC 234, GCC 104, GCC 157, GCC187, GCC 151, GCC 61, GCC 126, GCC 178, GCC 193 and GCC 106 in *Thevetia peruviana* (Table 1, 2). The reproducibility of the bands generated by these 10 primers was confirmed by replicating the amplification twice and if needed thrice. Only the bands showing reproducible amplification were considered for scoring and for further analysis. The total number of bands generated by ten amplifying primers was 72 and 64 with an average amplification of 7.2, 6.4 bands per primer in *Nerium oleander* and *Thevetia peruviana,* respectively and the average polymorphism generated by these bands in *Nerium oleander* and *Thevetia peruviana* was 23.67 % and 21.68 % respectively. The polymorphism information content was calculated based on the probability that two unrelated genotypes amplified from the test population will be placed into different polymorphic groups. The PIC (Polymorphic information content) ranges from 0.00 (GCC 112, GCC 51, GCC 121) to 0.262 (GCC 23); in case of *Nerium oleander* and from 0.00 (GCC 126, GCC 178, GCC 106) to 0.251 (GCC 157) in *Thevetia peruviana*.

Genetic relationship among the accessions and cluster analysis

For *Nerium oleander*, The Jaccard's pair wise similarity coefficient values ranged from 0.64 (1 and 2) to 0.97 (4 and 5) with an average of 81 %, for single primer based RAPD patterns and for *Thevetia peruviana,* the Jaccard's pair wise similarity coefficient values ranged from 0.65 (4 and 5) to 1.00 (1 and 2) with an average of 79 %, for single primer based RAPD patterns. The cluster was divided into 2 groups (A, B) (Figure 2, 4). It was observed that in both the plant samples, the *in vitro* grown callus developed from the explants procured from the *in vivo* grown mother plants (University of Rajasthan Jaipur, India), showed maximum similarity (97 % in *Nerium oleander*, 100 % in *Thevetia peruviana*), which indicated that the tissue cultured samples their source mother plant showed negligible or no difference at genotypic level.

DISCUSSION

RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. Despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labor (Bardakci *et al.,* 2001). Hence, the present investigation was undertaken to evaluate 5 genotypes of each plant species cultivated in different agro ecological areas (*in vivo)* along with *in vitro* grown callus. True-to-type clonal fidelity is one of the most important prerequisites in the micro propagation of any crop species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro* a better analysis of genetic stability of plantlets can be made by using RAPD markers which amplify different regions of the genome (Martins *et al*., 2004). Palombi and Damiano (2002) suggested the use of DNA amplification technique as advantageous in evaluating somaclonal variation while working on micro propagated plants. Hence, in the present study, PCR based techniques, RAPD were adopted for evaluation of clonal fidelity of *Nerium oleander* and *Thevetia peruviana*. Similar results using PCR - based techniques RAPD, have been found reported as one of the immensely useful technique in establishing the genetic stability of cultivated as well as *in vitro* regenerated culture of plant cells, tissues or organs in many other plant species viz., plants such as *Prunus dulcis* (Martins *et al*., 2004), apple (Modgil *et al*., 2005), *Pinus thunbergii* (Goto *et al*., 1998), cauliflower (Leroy *et al*., 2000) and *Digitalis obscura* (Gavida *et al*., 1996). RAPD fingerprinting was used for the detection of variety (Temiesak *et al.,* 1993) and clonal variation of plant species *in vitro* and *in vivo* (Wang *et al*., 2011, Arif *et al*., 2010). RAPD provides rapid results, is less time-consuming and less expensive (Arif and Khan, 2009), and gives information about genomic variability of species (Williams *et al*., 1990).

Table 1: List of Accession

Sample Type	Sample Type	Type of explant	Location
Nerium Oleander	*Thevetia peruviana*		
5	2	*In vivo* leaf	Department of Botany, University of Rajasthan, Jaipur
2	5	*In vivo* leaf	Chambal Garden, Kota
1	4	*In vivo* leaf	Agriculture University, Bikaner
3	3	*In vivo* leaf	Nursery, Phulera

Table 2: Similarity Matrix of 5 Samples of *N. oleander* computed from RAPD Data

Sample ID	Ng/ul	260/280
1	149.42	1.7
2	350.33	1.97
3	147.45	1.83
4	156.27	1.84
5	135.9	1.67

Table 3: DNA Bands of *N. oleander* accession compared with Primers

Primers	Size (bp)	Genotype				
		1	2	3	4	5
GCC23	2010	1	0	0	0	0
	1994	0	0	1	1	1
	1767	1	1	1	1	1
	1251	1	0	0	0	0
	1136	0	1	0	0	0
	1007	1	0	0	0	0
	945	0	1	1	1	1
	873	1	1	1	1	1
	810	1	0	1	0	0
	634	0	0	1	0	0
	596	1	1	1	1	1
GCC151	1842	0	1	0	0	0
	1302	1	1	1	1	1
	1256	0	0	0	0	0
	926	1	0	1	0	1
	893	0	0	0	0	0
	674	1	0	1	1	1
	610	1	1	1	1	1
	518	0	1	0	0	0
	461	1	0	0	0	0
GCC104	1812	1	1	1	1	1
	1465	1	0	1	1	1
	1222	0	1	0	0	0
	1035	1	1	1	1	1
	776	1	1	1	1	1
	569	0	1	0	0	0
	321	1	1	1	1	1
	201	1	1	1	1	1
GCC121	2112	1	1	1	1	1
	1900	1	1	1	1	1
	1750	1	1	1	1	1
	1460	1	1	1	1	1
	1310	1	1	1	1	1
	1130	1	1	1	1	1
	970	1	1	1	1	1
	760	1	1	1	1	1
	550	1	1	1	1	1
	457	1	1	1	1	1
	310	1	1	1	1	1
GCC51	650	1	1	1	1	1
	554	1	1	1	1	1
	420	1	1	1	1	1
GCC112	1910	1	1	1	1	1
	1780	1	1	1	1	1
	1505	1	1	1	1	1
	1007	1	1	1	1	1
	940	1	1	1	1	1
	570	0	0	0	0	0
	430	1	1	1	1	1
GCC135	3400	1	1	1	1	1
	2450	1	1	1	1	1
	2002	1	1	1	1	1
	1807	0	1	0	0	0
	810	1	1	1	1	1
	795	1	1	1	1	1
	680	1	1	1	1	1
GCC103	1310	0	0	0	0	0
	1108	0	0	0	0	0
	820	1	0	0	0	0
	740	1	1	1	1	1
	610	1	1	1	1	1
	570	0	0	0	0	0
GCC184	1600	1	1	1	1	1
	1380	0	0	0	0	0
	890	1	1	1	1	1
	784	0	0	0	0	0
	650	1	1	1	0	0
GCC61	1400	1	1	1	1	1
	1289	0	1	0	0	0
	1035	1	1	1	1	1
	810	1	1	1	1	1
	621	1	1	1	1	1

Table 4: DNA Bands of *N. oleander* accession compared with Primers by PIC value

Primers	Sequences (5'	Total No. of bands (a)	Total No. of polymor-phic bands(b)	Polymor-phism(b/a*100)
GCC23	CCCGCCTTCC	11	8	72.73
GCC151	GCTGTAGTGT	9	5	55.56
GCC104	GGGCAATGAT	8	3	37.50
GCC121	ATACAGGGAG	11	0	0.00
GCC51	CTACCCGTGC	3	0	0.00
GCC112	GCTTGTGAAC	7	0	0.00
GCC135	AAGCTGCGAG	7	1	14.29
GCC103	GTGACGCCGC	6	1	16.67
GCC184	CAAACGGCAC	5	1	20.00
GCC61	TTCCCCGACC	5	1	20.00
Total		**72**	**20**	**Average= 23.67**

Table 5: DNA Bands of *N. oleander* accession compared with Primers by PIC value

Primer	Size (bp)	No of 1	No. of 0	F = No. of Band by Presence	E = No. of Band by Absence	PIC = 1-(F*F) + (E*E)	PIC = 2F (1-F)	Average PIC
GCC23	2010	1	4	0.2	0.8	0.32	0.32	0.262
	1994	3	2	0.6	0.4	0.48	0.48	
	1767	5	0	1	0	0	0	
	1251	1	4	0.2	0.8	0.32	0.32	
	1136	1	4	0.2	0.8	0.32	0.32	
	1007	1	4	0.2	0.8	0.32	0.32	
	945	4	1	0.8	0.2	0.32	0.32	
	873	5	0	1	0	0	0	
	810	2	3	0.4	0.6	0.48	0.48	
	634	1	4	0.2	0.8	0.32	0.32	
	596	5	0	1	0	0	0	
GCC151	1842	1	4	0.2	0.8	0.32	0.32	0.196
	1302	5	0	1	0	0	0	
	1256	0	5	0	1	0	0	
	926	3	2	0.6	0.4	0.48	0.48	
	893	0	5	0	1	0	0	
	674	4	1	0.8	0.2	0.32	0.32	
	610	5	0	1	0	0	0	
	518	1	4	0.2	0.8	0.32	0.32	
	461	1	4	0.2	0.8	0.32	0.32	
GCC104	1812	5	0	1	0	0	0	0.120
	1465	4	1	0.8	0.2	0.32	0.32	
	1222	1	4	0.2	0.8	0.32	0.32	
	1035	5	0	1	0	0	0	
	776	5	0	1	0	0	0	
	569	1	4	0.2	0.8	0.32	0.32	
	321	5	0	1	0	0	0	
	201	5	0	1	0	0	0	
GCC121	2112	5	0	1	0	0	0	0.000
	1900	5	0	1	0	0	0	
	1750	5	0	1	0	0	0	
	1460	5	0	1	0	0	0	
	1310	5	0	1	0	0	0	
	1130	5	0	1	0	0	0	
	970	5	0	1	0	0	0	
	760	5	0	1	0	0	0	
	550	5	0	1	0	0	0	
	457	5	0	1	0	0	0	
	310	5	0	1	0	0	0	
GCC51	650	5	0	1	0	0	0	0.000
	554	5	0	1	0	0	0	
	420	5	0	1	0	0	0	
GCC112	1910	5	0	1	0	0	0	0.000
	1780	5	0	1	0	0	0	
	1505	5	0	1	0	0	0	
	1007	5	0	1	0	0	0	
	940	5	0	1	0	0	0	
	570	0	5	0	1	0	0	
	430	5	0	1	0	0	0	
GCC135	3400	5	0	1	0	0	0	0.046
	2450	5	0	1	0	0	0	
	2002	5	0	1	0	0	0	
	1807	1	4	0.2	0.8	0.32	0.32	
	810	5	0	1	0	0	0	
	795	5	0	1	0	0	0	
	680	5	0	1	0	0	0	

GCC103	1310	0	5	0	1	0	0	0.053
	1108	0	5	0	1	0	0	
	820	1	4	0.2	0.8	0.32	0.32	
	740	5	0	1	0	0	0	
	610	5	0	1	0	0	0	
	570	0	5	0	1	0	0	
GCC184	1600	5	0	1	0	0	0	0.096
	1380	0	5	0	1	0	0	
	890	5	0	1	0	0	0	
	784	0	5	0	1	0	0	
	650	3	2	0.6	0.4	0.48	0.48	
GCC61	1400	5	0	1	0	0	0	0.064
	1289	1	4	0.2	0.8	0.32	0.32	
	1035	5	0	1	0	0	0	
	810	5	0	1	0	0	0	
	621	5	0	1	0	0	0	

Table 6: JACCARD'S coefficient similarity of *N. oleander* accession

	1	**2**	**3**	**4**	**5**	
1	1.00					
2	0.65	1.00				
3	0.81	0.71	1.00			
4	0.78	0.77	0.91	1.00		
5	0.81	0.75	0.94	0.97	1.00	
Total	3.04	2.23	1.85	0.97		0.81

Table 7: Similarity matrix of 5 Samples of *T. peruviana* computed from RAPD Data

Sample ID	**ng/ul**	**260/280**
1	115.68	1.52
2	159.73	1.78
3	125.24	1.67
4	278.09	1.94
5	322.12	2.01

Table 8: DNA Bands of *T. peruviana* accession compared with Primers

Primers	**Size (bp)**	**Genotype**				
		1	**2**	**3**	**4**	**5**
GCC234	1989	1	1	1	1	0
	1473	1	1	1	1	1
	1280	1	1	1	1	1
	765	1	1	1	1	1
	473	1	1	1	1	1
GCC157	3425	1	1	1	1	1
	1466	1	1	1	1	0
	1235	0	0	1	1	0
	1184	1	1	0	1	0
	1065	1	1	1	1	1
	872	0	0	1	1	1
	814	1	1	1	1	1
	797	0	0	1	0	1
	564	1	1	1	1	1
	485	1	1	1	1	1
	447	1	1	1	1	1
	424	0	0	0	0	1
	412	0	0	1	0	1
	390	1	1	0	1	0
GCC104	1872	1	1	1	1	1
	1267	1	1	1	1	1
	1035	1	1	1	1	1
	765	1	1	1	1	1
	564	1	1	1	0	1
	472	1	1	1	1	1
	352	1	1	1	1	1
GCC187	1560	0	0	0	0	1
	1300	0	0	1	0	1
	765	1	1	0	1	0
	634	1	1	1	1	1
	530	1	1	1	1	1
	430	1	1	1	1	Contd...
	397	1	1	1	1	1
GCC151	1003	1	1	1	1	1
	997	1	1	1	1	1
	840	1	1	1	1	1
	630	1	1	1	0	1

GCC61	1490	1	1	1	1	1
	1105	1	1	1	0	1
	1095	1	1	1	1	1
	810	1	1	1	1	1
	780	1	1	1	1	1
	630	1	1	1	1	1
	595	1	1	1	1	1
	470	1	1	1	1	1
GCC126	1703	1	1	1	1	1
	1309	1	1	1	0	1
	953	0	0	0	0	0
	630	0	0	0	0	0
	510	1	1	1	1	1
GCC178	2000	1	1	1	1	1
	1710	1	1	1	1	1
	1003	1	1	1	1	1
	905	1	1	1	1	1
	810	1	1	1	1	1
	730	0	0	0	0	0
	500	1	1	1	1	1
GCC193	934	1	1	1	1	1
	537	1	1	0	1	0
	450	1	1	1	1	1
	430	1	1	1	1	1
GCC106	3370	1	1	1	1	1
	3100	1	1	1	1	1
	631	1	1	1	1	1

Table 9: Details of Primers, Bands and Polymorphism *T. peruviana* revealed by 10 Primers used RAPD-PCR

Primers	Sequences	Total No. of bands (a)	Total No. of polymor-phic bands(b)	Polymor-phism (b/a*100)
	(5’			
GCC234	TCCACGGACG	5	1	20.00
GCC157	CGTGGGCAGG	14	8	57.14
GCC104	GGGCAATGAT	7	1	14.29
GCC187	AACGGGGGAG	7	3	42.86
GCC151	GCTGTAGTGT	4	1	25.00
GCC61	TTCCCCGACC	8	1	12.50
GCC126	CTTTCGTGCT	5	1	20.00
GCC178	CCGTCATTGG	7	0	0.00
GCC193	TGCTGGCTTT	4	1	25.00
GCC106	CGTCTGCCCG	3	0	0.00
Total		64	17	21.68

Table 10: DNA Bands of *T. peruviana* accession compared with Primers by PIC value

Primers	Size (bp)	No of 1	No. of 0	F = No. of Band by Presence	E = No. of Band by Absence	PIC = 1-(F*F) + (E*E)	PIC = 2F(1-F)	Average PIC
GCC234	1989	4	1	0.8	0.2	0.32	0.32	0.064
	1473	5	0	1	0	0	0	
	1280	5	0	1	0	0	0	
	765	5	0	1	0	0	0	
	473	5	0	1	0	0	0	
GCC157	3425	5	0	1	0	0	0	0.251
	1466	4	1	0.8	0.2	0.32	0.32	
	1235	2	3	0.4	0.6	0.48	0.48	
	1184	3	2	0.6	0.4	0.48	0.48	
	1065	5	0	1	0	0	0	
	872	3	2	0.6	0.4	0.48	0.48	
	814	5	0	1	0	0	0	
	797	2	3	0.4	0.6	0.48	0.48	
	564	5	0	1	0	0	0	
	485	5	0	1	0	0	0	
	447	5	0	1	0	0	0	
	424	1	4	0.2	0.8	0.32	0.32	
	412	2	3	0.4	0.6	0.48	0.48	
	390	3	2	0.6	0.4	0.48	0.48	
GCC104	1872	5	0	1	0	0	0	0.046
	1267	5	0	1	0	0	0	
	1035	5	0	1	0	0	0	
	765	5	0	1	0	0	0	
	564	4	1	0.8	0.2	0.32	0.32	
	472	5	0	1	0	0	0	
	352	5	0	1	0	0	0	
GCC187	1560	1	4	0.2	0.8	0.32	0.32	0.183

	1300	2	3	0.4	0.6	0.48	0.48	
	765	3	2	0.6	0.4	0.48	0.48	
	634	5	0	1	0	0	0	
	530	5	0	1	0	0	0	
	430	5	0	1	0	0	0	
	397	5	0	1	0	0	0	
GCC151	1003	5	0	1	0	0	0	0.080
	997	5	0	1	0	0	0	
	840	5	0	1	0	0	0	
	630	4	1	0.8	0.2	0.32	0.32	
GCC61	1490	5	0	1	0	0	0	0.040
	1105	4	1	0.8	0.2	0.32	0.32	
	1095	5	0	1	0	0	0	
	810	5	0	1	0	0	0	
	780	5	0	1	0	0	0	
	630	5	0	1	0	0	0	
	595	5	0	1	0	0	0	
	470	5	0	1	0	0	0	
GCC126	1703	5	0	1	0	0	0	0.000
	1309	5	0	1	0	0	0	
	953	0	5	0	1	0	0	
	630	0	5	0	1	0	0	
	510	5	0	1	0	0	0	
GCC178	2000	5	0	1	0	0	0	0.000
	1710	5	0	1	0	0	0	
	1003	5	0	1	0	0	0	
	905	5	0	1	0	0	0	
	810	5	0	1	0	0	0	
	730	5	0	1	0	0	0	
	500	5	0	1	0	0	0	
GCC193	934	3	2	0.6	0.4	0.48	0.48	0.120
	537	5	0	1	0	0	0	
	450	5	0	1	0	0	0	
	430	5	0	1	0	0	0	
GCC106	3370	5	0	1	0	0	0	0.000
	3100	5	0	1	0	0	0	
	631	5	0	1	0	0	0	

Table 11: JACCARD'S coefficient similarity of *T. peruviana* accession

	1	**2**	**3**	**4**	**5**	
1	1.00					
2	1.00	1.00				
3	0.76	0.76	1.00			
4	0.87	0.87	0.80	1.00		
5	0.68	0.68	0.83	0.65	1.00	
Total	3.31	2.31	1.63	0.65		0.79

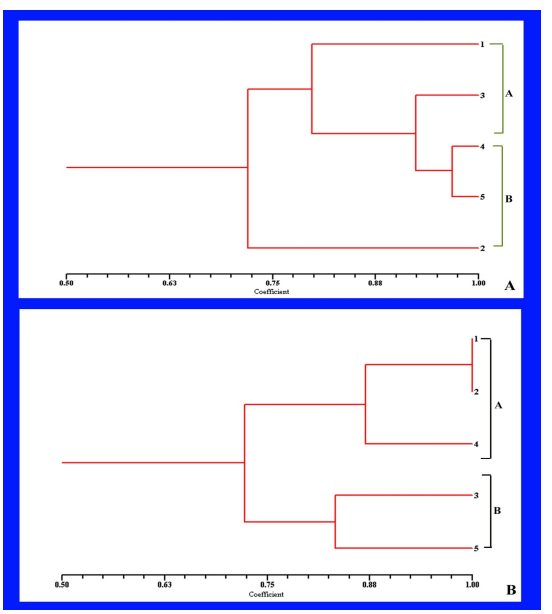

Figure 1: Dendrogram showing Relationship among Five Genotypes of *Nerium oleander* Generated by UPGMA analysis
Figure 2: Dendrogram showing Relationship among Five Genotypes of *Thevetia peruviana* Generated by UPGMA analysis

Figure 3: Details of Primers, Bands and Polymorphism of Both *N. oleande* and *T. peruviana* revealed by 10 Primers used RAPD- PCR

Therefore, our findings provide guidance for the identification of these plant species growing in an extreme arid environment as well as *in vitro* conditions, to help in their subsequent management and utilization in sustainable ways to combat human and natural pressures on these valuable natural resources.

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