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GENETIC DIVERSITY AND POPULATION STRUCTURE AMONG *Grewia tenax* GENOTYPES IN SUDAN USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

RAMI S.HABEBALLA[1, 2*], RASHIED S.MODAWI[3], MUTASIM M.KHALAFLA[2, 4] AND NADA B.HAMZA[2]

[1]Department of Basic Health Sciences, faculty of pharmacy, Northern Border University, Kingdom of Saudi Arabia, Rafha

[1]Department of Molecular Biology, [2]Commission for Biotechnology and Genetic Engineering, National Centre for research, Sudan

[3]Department of Biology and Biotechnology, Faculty of Science and Technology, Al Neelain University, Sudan

[4]Faculty of public Health and Health Informatics, Umm Al Qura University, Makah Al Mukarramah, Saudi Arabia

***Corresponding author: Department of Basic Health Sciences, faculty of pharmacy, Northern Border University, Kingdom of Saudi Arabia, Rafha: Email:ramialsir@yahoo.com, ramialsir@gmail.com, Mobile +966 544759869**

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ABSTRACT

Random Amplified polymorphic DNA (RAPD) markers were used to characterize the genetic diversity and relationships within and among *Grewia tenax* populations. 144 *Grewia spp* genotypes were collected from different localities of Sudan extending from the western, central and eastern Sudan with different ecological zones. DNA was isolated from *Grewia spp* genotypes according to modified phenol: chloroform: isoamyl alchohol. A total of 14 RAPD primers were selected and used to evaluate the degree of polymorphism and genetic relationships within and among all the *Grewia spp* under study. Total of 119 amplified

fragments were distinguished across the selected primers and the statistical analysis showed 112 polymorphic bands among the 144 genotypes with an average of 8 polymorphic bands per primer. The maximum numbers of fragment bands were produced by the primer OPA- 17 (13) with 100% polymorphism while the minimum numbers of fragments were produced by the primers PF-08 (6) with 100% polymorphism, OPA-04(6) with 83% polymorphism, OPF-15 (6) with 100% polymorphism and OPA-09 (6) bands with 83% polymorphism. The total genetic diversity (He) and Shannon's diversity information index (I) for the populations were 0.340 and 0.234 respectively. Analysis of molecular variance within populations revealed higher genetic diversity (71%) of the total genetic diversity compared to 29% among populations. This research could be a good step towards to characterize the genetic diversity found within the *Grewia tenax* genotypes. High level of polymorphism within individuals suggested that RAPD technique can be useful tool for characterization and maintenance of *Grewia* genotypes for efficient selection of parents for breeding. Such information about the genetic relation is valuable for breeders that can be used to improve adaptive and agronomic traits through marker- assisted selection techniques.

Keywords: Grewia tenax, genetic diversity, RAPD, GenAlEx, AMOVA, Unweighted Pair group method with arithmetic mean (UPGMA).

INTRODUCTION

Grewia tenax (Forssk.) Fiori belonging to the *Malvaceae* family is a multi-stemmed fruit shrub with manifold uses throughout the tropics and subtropics [1]. It has been used for decades for the preparation of traditional medicines. *Grewia tenax* is considered as a typical plant species which can tolerate seasonal drought and withstand temperatures of more than 50°C [2]. Moreover, *Grewia tenax* is also known as dune fixing species because of its dense fast growing root system [3]. It is a deciduous fruit-producing shrub or small tree that may attain a height of 1 to 3 m.

As result to the overexploitation of *Grewia tenax* and lack of regeneration, its wild stands became increasingly threatened so the fruit sources and natural gene pool were exhausted [2]. *Grewia tenax* grows wild at low elevations throughout the western Sahelian zone (Mali, Mauritania, Niger, Nigeria and Senegal), the eastern Sahelian zone (Djibouti, Eritrea, Ethiopia, Kenya, Somalia and Sudan), northern Africa (Algeria and Morocco) as well southern

Africa (Botswana, Namibia, Transvaal and South Africa). It is also found in the Arabian Peninsula and from Iran to India [1]. In Sudan and South of Sudan, *G. tenax* is found in Bahr El Gazal, Blue Nile, Darfur, Equatoria, Kassala, Khartoum, Kordofan, Upper Nile and White Nile province [4].

The fruit known locally in the Sudan as "Gudaim" is a rich source of carbohydrates, protein, vitamins and minerals and constitutes important contributors to improving the nutritional contents of rural and urban people in Sudan [5].It is a very important wild fruit-producer and prime supplier for food especially during time of food shortages [6].

G. tenax fruits were considered for a long time as a simple, naturally available food and medication against iron-deficiency anemia and fatigue [7, 8, 2].Anemia, which is one of the top ten death-causing disorders in developing countries, not related only to malnutrition and poverty, but also to the free radicals activities[9, 10]. *Grewia tenax* extracts were report to have effects on the regulation of iron digestive transfer and absorption [8].

Genetic variation within and among populations can be investigated by employing biochemical markers (isozymes/allozymes), direct DNA sequencing and using molecular (DNA) markers [11]. Until recently, research on the genetics of tropical trees was confined largely to allozyme studies of the genetic structure of adults in continuous forests [12, 13]. The first DNA marker exploited is referred to as Restriction Fragment Length polymorphism [14]. The recent molecular techniques such as Random Amplified polymorphic DNA [15], Inter Simple Sequence Repeat polymorphism [16], Microsatellites [17], Amplified Fragment Length polymorphism [18] as well as Inverse Sequence-Tagged Repeat [19], which involve the Polymerase Chain Reaction (PCR).In this technique amplification of the fragments of genomic DNA is conducted using a heat-resistant DNA polymerase (Taqpolymerase), primers and deoxyribonucleotide triphosphates at high temperatures [20]. The use of molecular markers in the investigation of genetic variation is getting a wide acceptance and broad application in fields such as phylogeny, taxonomy, ecology, genetics and breeding [11].

The general objectives of the study is to determine the genetic variation in populations of *Grewia tenax* found at different altitudes and geographical locations ranging from scattered to continuous populations over a wide geographical

distance of Sudan, by employing DNA marker systems.

MATERIALS AND METHODS

Collection Sites and laboratory

Leaves collected from 12 locations representing the 12 populations each population consisting of 12 samples of natural population considered to represent the geographical range of *Grewia tenax*. Samples were carried to the laboratory in brown paper bags and kept in the deep freezer (− 20°C) for future DNA extraction. The Selection of parameters for leaves collection was based on productivity, type of soil and location. The locations and details of leaves are listed in Table 1 and Table 2. All samples were collected from natural, healthy and productive *Grewia tenax* trees. Total genomic DNA was isolated in laboratory of Basic health Sciences, Faculty of pharmacy using the Dellaporta method [21] with slight modification made in the buffer concentrations.

Table1: longitudes, latitudes and location of *Grewia tenax* samples.

Location	Longitude	Latitude
Elobied, 3 populations	30° 13'E	13° 11'N
Khor alabyad	30° 12'E	30° 10'N
Al-Ain	30° 20'E	13° 00'N
Aldamazin	34° 21'E	11° 46'N
Zalingei	23° 29' E	12° 54'N
Abuharaz	33° 51' E	14° 48'N
Almnzfa	29° 32' E	14° 22'N
Khartoum	32° 33'E	15° 30'N
Shambat-Bahry	32° 38' E	15° 38' N
Garsilla	25° 14'E	11° 27'N

Table 2: Sources of *Grewia tenax* leaves, locations in Sudan and soil types at the site

Sample No.	Name (Area)	Site of collection	Location	Type of soil
1-12	Abuhraz	Northern Kordofan	Western Sudan	Sandy clay
13-24	Almnzfa	Northern Kordofan	Western Sudan	sand
25-36	Elobaid1	Northern Kordofan	Western Sudan	sand
37-48	Garsilla	NorthernKordofan	Western Sudan	sand
37-48	Garsilla	NorthernKordofan	Western Sudan	sand
49-60	Zalingei	Zalingei	Northern West of Nyala	Sand
61-72	Elobaid2	Northern Kordofan	Western Sudan	sand
73-84	Khoralbyed	Northern Kordofan	Western Sudan	Alluvial
73-84	Khoralbyed	Northern Kordofan	Western Sudan	Alluvial
85-96	Khoralbyed	Northern Kordofan	Western Sudan	Sandy clay
97-108	Elobaid3	Northern Kordofan	Western Elobaid	Sandy clay
109-120	ALdamazin	Blue Nile	Southern blue Nile	clay
121-132	Khartoum	Teacher houses	Southern Khartoum	Clay
133-144	Shambat-Bahry	Teacher houses	Northern Khartoum	Clay

DNA Extraction

Sample preparation

Fresh young and healthy leaves were collected from plant samples (0.5 g) and kept in aluminum foil under cool conditions till use. Samples were placed into mortar grinders and covered with dry ice powder until it thawed followed by grinding to fine powder with vigorous pulverizing at intervals of 20 to 30 seconds. Ground green dry powders were immediately used for DNA isolation.

DNA extraction and purification

DNA isolation was based on modified phenol: chloroform: isoamyl alchohol (24:25:1) protocol as described by[21]. The modification was made with the intention to improve the quantity and the quality of the DNA. In this method the fine powder plant materials (0.5g) were immediately transferred into 15 ml Falcon tubes containing 6 ml of pre-warmed lysis solution. Tubes containing the samples were then incubated in water path at 65 °C with gentle shaking for 30 min and left to cool at room temperature for 5 min. 3ml of phenol: chloroform: (25:24) added to each tube and the phases were mixed gently for 5 min at room temperature to make homogeneous mixture. The cell debris was removed by centrifugation at 5000 rpm for 15 min and the resulted clear aqueous phases (containing DNA) were transferred to new sterile tubes.

The transferred aqueous phase was mixed thoroughly with equal volume of chloroform: isoamyl alchohol (24:1) followed by centrifugation at 5000 rpm for 5min. The step of the chloroform: isoamyl alchohol extraction was repeated twice and the final supernatants were transferred to sterile 1.5 ml Eppendorf tubes (400ul/ tube). The nucleic acids in the aqueous phase was precipitated by adding equal volume of cooled absolute ethanol. The contents were mixed gently for 5 min by inversion manually and collected by

cooled centrifugation (5°C) at 8000 rpm for 3 min.

The formed DNA pellet was washed twice with 70% ethanol, and the ethanol was discarded after spinning with flash centrifugation. The remained ethanol was removed by leaving the pellet to dry at room temperature. The pellet was dissolved in TE buffer. The extracted DNA samples were observed under UV illumination after staining with (Ez- Vission cat number 10450003-1).

DNA Quantification

According to Sambrook et al (1989) [22], the purity and the concentration of the DNA were assessed. The DNA isolated was quantified by using Lambda UV-vis spectrophotometer. First the absorbance was set to zero by using 50 µl of Tris EDTA buffer in the cuvette. The cuvette was properly washed with distilled water, 2 µl of the isolated DNA added to 48 µl of TE buffer, the ratio at 260/280 nm was recorded following the amount of DNA in concentration of ng/µl. This quantification of isolated DNA provided with concentration of DNA present in a particular sample and also the 260/280 nm ratio was estimated to find out purity of DNA. The quantified samples were brought to the final working concentration of DNA of 5ng/µl, which will then be used for the RAPD and analysis.

RAPD analysis

Primers

For standardization of the amplification conditions a total of twenty decamer oligonucleotide primers were used for amplification of the extracted cellular DNAs. Primers were selected according to the reproducibility of their amplification product. Primers used in this study were obtained from Operon Technologies Inc., USA (Table 3).

Standardization of concentration of DNA for RAPD

For standardizing the concentration of template DNA, PCR amplification was performed with different DNA concentrations using previously standardized master-mix concentrations. Five different concentrations of DNA were used which were 5 ng, 10 ng, 15 ng, 20 ng and 25 ng, for obtaining the maximum number of amplification products. It was found that maximum number of amplification products was observed at 20 ng of template DNA.

Standardization of PCR amplification conditions

Tests were performed for standardizing polymerase chain reaction amplification conditions mainly the annealing temperature.

PCR amplification conducted at different annealing temperatures i.e. 36°C, 37°C, 38°C using standard concentrations of various components of reaction mixture. It was found that maximum number of amplification products was observed at 20 ng of template.

Polymerase chain reaction (PCR) mixture

The PCR reaction mixtures were prepared in 25µl volumes containing 2.5µ of buffer (B), 3µl $MgCl_2$ (25mM), 0.25µl dNTP's (200µM), 2µl random primer (10 pmol/µl), 0.5µl Taq polymerase 5U/µl and 1µl of extracted DNA (20 ng).The mixture was made up to 25 µl by addition of sterilized distilled water. The reagents were mixed thoroughly in 2ml microcentrifuge tube and vortexed for 5seconds. 24µl of mixture was distributed to each PCR tube and 1µl of template DNA (20 ng/µl) was added to each tube for each amplification reaction in thermal cycler (Real- time PCR, Bio- Rad CFX96).

DNA amplification

PCR conditions used for RAPD amplification included initial denaturation for 3 min at 94°C followed by45 cycles of amplification (denaturation at 92ºC for 45seconds, annealing of primer at 36ºC for 1min and primer amplification at 72ºC for 2 min) and final extension at72ºC for 10 min. The PCR machine was adjusted to hold the product at 4ºC.

Electrophoresis using agarose gel

Amplification products were separated on 2 per cent agarose gel using 1XTBE buffer (Tris HCI pH 8.0, Boric Acid, Ethylene diamine tetra- Acetic). Horizontal gel electrophoresis apparatus (Consort-UK). Ez-vission dye was used as intercalating agent. 5µl of RAPD amplification products were mixed with 1µl of Ez-vission dye and loaded on to the gel. Gel was run according to 5v/cm of the length of gel till the bands separate. 1 Kb DNA ladder (250 - 1000bp, cat number :DM010-R500) was used as standard in the first well of the gel.

Data analysis

Viewing of amplified DNA

The gel was viewed under the UV light using Gel Documentation system and was photographed.

Scoring of bands

The amplified bands were visualized using Gel Documentation system. Further the bands were scored for percentage polymorphism for each set of primer amplified product using statistical software package STATISTCA SPPS ver.9 and GenALEx ver. 6.5 software [23].

For each primer, the number of polymorphic and monomorphic bands was determined.

Bands that are clearly visible in at least one genotype were scored (1) for present, 0 for absent and entered into data matrix. Fragment size was estimated by interpolation from the migration distance of marker fragments. Percentage of polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. The genetic dissimilarity (D) matrix among genotypes was estimated according to Nei andLei (1979)[24]. Coefficient of similarity trees were produced by clustering the similarity data with the unweighted pair group method using STATISTCA and GenALEx 6.5software. The similarity coefficient was used to construct a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) according to[25].Banding pattern of DNA fragments, number of private alleles, gene diversity, Shannon's information index [26] as well as Pairwise PhiPT values were considered between groups. Pairwise PhiPT values were computed with 9999 permutations to determine probability and level of significance of the variance components. The RAPD data set is considered for analysis of molecular variance (AMOVA) for the groups according to [27]. Principal coordinates analysis (PCoA) was performed with the GenAlEx 6.5 software, using unbiased Nei genetic distances.

RESULTS AND DISCUSSION

DNA extraction

During our experimentation we observed that minor alterations in the quality of the targeted DNA can alter the resolution of the RAPD pattern, thus we followed a modified phenol-chloroform method to optimize conditions for PCR amplification. The DNA extracted by the modified method was found to be a good template for PCR amplification that increases the reproducibility and efficiency of RAPD's as molecular markers (Figures 1- 4).

Band scoring and cluster analysis

The reproducibility of the amplification product was tested on template DNA. Most of the amplification reactions were duplicated. Only bands that were consistently reproduced across amplifications were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. Fourteen RAPD informative primers were selected and used to evaluate the degree of polymorphism within all the tree species of *Grewia tenax* (Table 3).

RAPD analysis

Of the 20 primers tested with the 114 genotypes of *Grewia tenax*, 14 primers (70%) showed at least one consistent polymorphic band. The 14 primers were selected and used to evaluate the degree of polymorphism and genetic relationships within and between all the *Grewia spp* under study. The selected primers generated distinctive products in the range of 250 – 4000 bp.A total of 119 amplified fragments were distinguished across the selected primers and the statistical analysis showed 112 polymorphic bands among the 144 genotypes with an average of 8polymorphic bands per primer. The maximum numbers of fragments bands were produced by the primer OPA-17 (13) with 100% polymorphism while the minimum numbers of fragments were produced by the primers PF-08 (6) with 100% polymorphism, OPA-04(6) with 83% polymorphism,OPF-15 (6) with 100% polymorphism and OPA-09 (6) bands with 83% polymorphism.

Table 3: Polymorphism detected by the use of 14 RAPD primers on 144 genotypes of *Grewia tenax*

Primer code	Sequence of primer (5'-3')	Total number of bands	Unique band	Number of polymorphic bands	Number of monomorphic bands	% of Polymorphic bands
PF-08	GGGATATCGG	6	0	6	0	100%
OPA-04	AATCGGGCTG	6	1	5	0	83%
OPA-05	AGGGGTCTTG	11	0	10	1	91%
OPA-09	GGGTAACGCC	6	0	5	1	83%
OPA-17	GACCGCTTGT	13	0	13	0	100%
OPC-04	CCGCATCTAC	7	0	6	1	86%
OPC-11	AAAGCTGCGG	9	0	9	0	100%
OPC-13	AAGCCTCGTC	10	0	10	0	100%
OPF-12	ACGGTACCAG	7	0	7	0	100%
OPF-13	GGCTGCAGAA	7	0	7	0	100%
OPF-15	CCAGTACTCC	6	0	6	0	100%
OPG-10	AGGGCCGTCT	11	0	11	0	100%
OPA-08	CAGCGACTGT	9	0	9	0	100%
OPO-20	ACACACGCTC	11	1	8	2	73%
Total		119	2	112	5	1316
Average		11.9	0.2	8	0.	94%

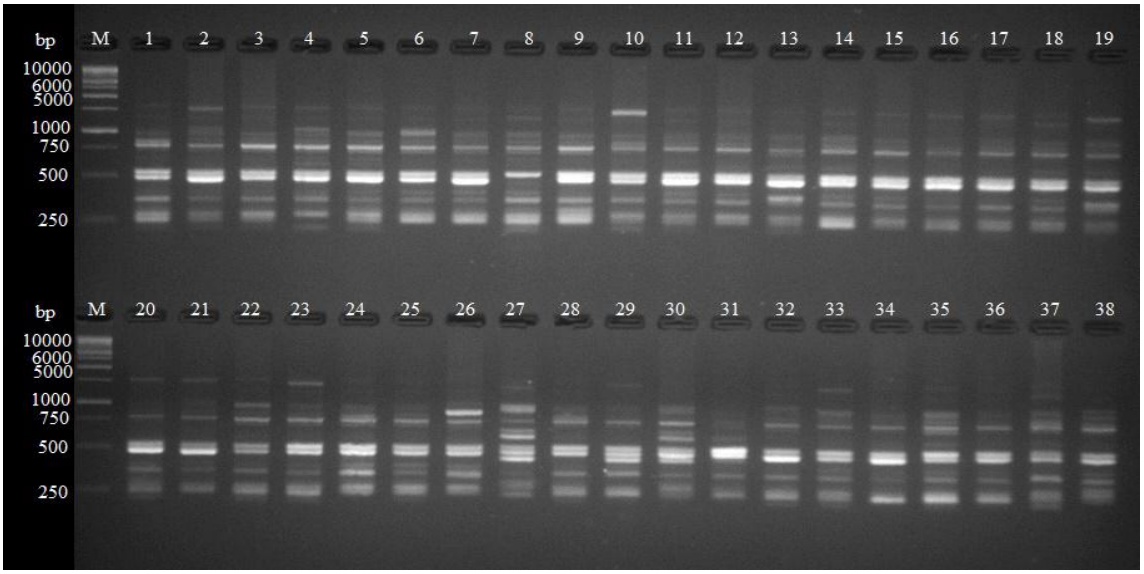

Figure 1: The RAPD profile of *Grewia spp* genotypes produced with primer OPO-20 (Lane M is a 1 kb ladder and lanes 1 to 38 represent *Grewia spp* genotypes).

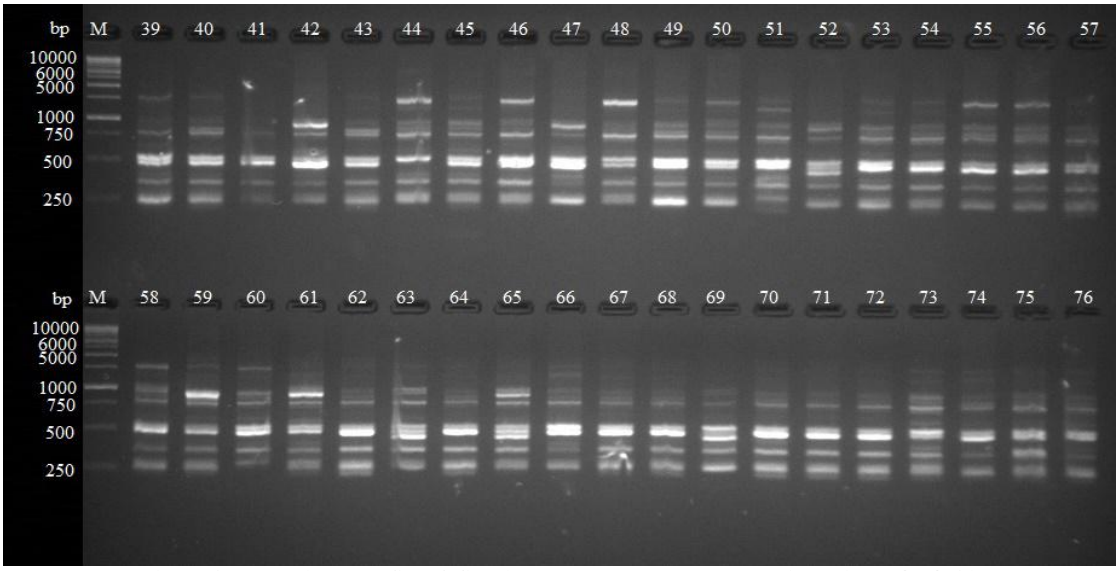

Figure 2: The RAPD profile of *Grewia spp* genotypes produced with primer OPO-20 (Lane M is a 1 kb ladder and lanes 39 to76 represent *Grewia spp* genotypes).

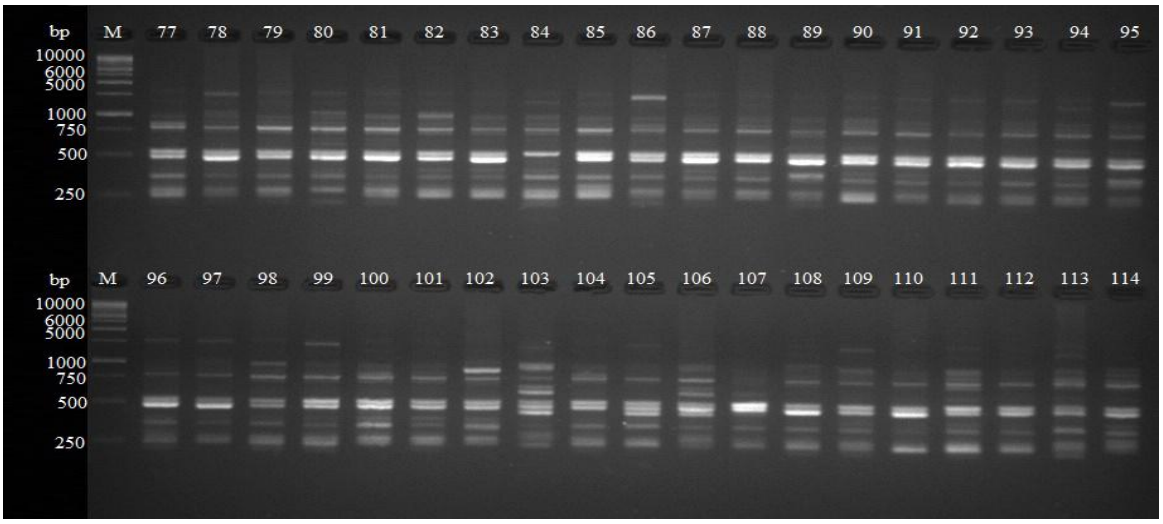

Figure 3: The RAPD profile of *Grewia spp* genotypes produced with primer OPO-20 (Lane M is a 1 kb ladder and lanes 77 to 114 represent *Grewia spp* genotypes).

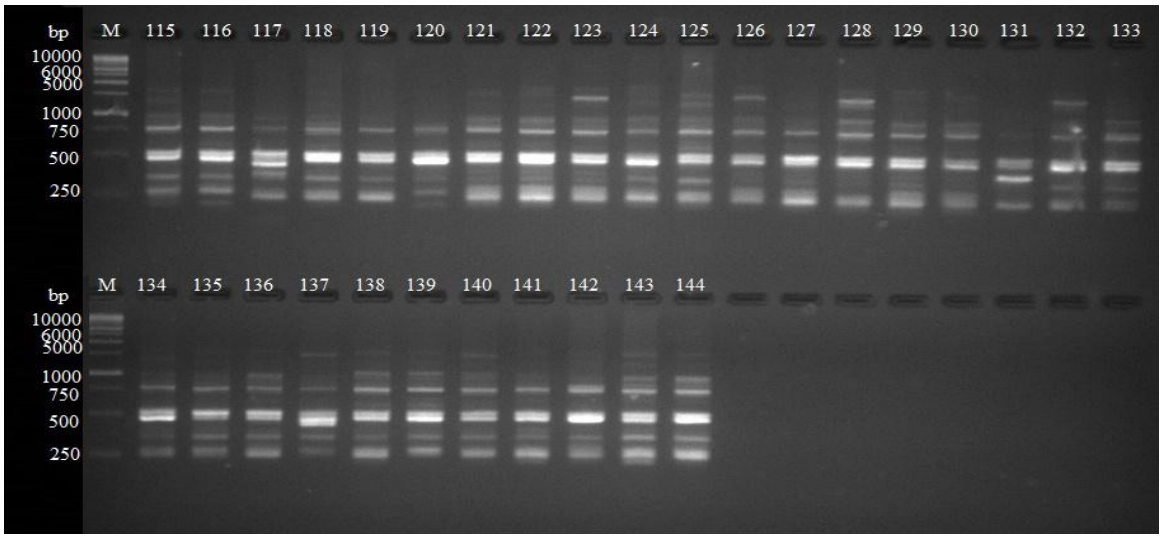

Figure 4: The RAPD profile of *Grewia spp* genotypes produced with primer OPO-20 (Lane M is a 1 kb ladder and lanes 115 to 144 represent *Grewia spp* genotypes).

Genetic Diversity within populations of *Grewia* Genotypes

The evaluation of twelve *Grewia* populations (H_e) detected with 14 RAPD primers resulted in overall *Grewia* genotypes diversity recorded at 0.234 (Table 4).

Based on pair-wise population analysis, Abuhrazwas found to be the most diverse population with average Nei's gene diversity and Shannon index values of 0.351 and 0.531, whereas Elobaid 2 was the least diverse followed by Aldamazin (0.216 and 0.155) and Shannon index of (0.318 and 0.233) respectively (Table 4). The different (Na) and effective number (Ne) of alleles also followed the same trend and were highest for Abuharaz and lowest for Elobaid 2 and Aldmazin populations (Table 4). The proportion of polymorphic loci for the present set of populations ranged from 44.54% (Elobaid 2) to Abuharaz with average 60.64% polymorphism.

Polymorphism (44.54%) was detected for Elobaid 2 population was lower compared to the previous results of [28] who reported 87.78% polymorphism on screening of twenty five primers from 119 random primers in Joute.

The maximum dissimilarity (0.58) was observed between samples (Abuhraz 2 and Abuhraz 4) while the minimum dissimilarity (0.05) was recorded between (Elobaid 62 and Elobaid 65), (Elobaid 62 and Elobaid 64).

Pair-wise distance analysis (Nei genetic distance) between populations (Table 5) ranged from 0.328 (between Abuhraz and Shambat) to 0.069 (between Almnzfaand Abuhraz). The highest distances were observed between the populations of Shambat. The distances between the populations of *Grewia spp* were much lower indicating the genetic relatedness of the various geographic populations which may be caused by the high rates of gene flow due to the exchange of *Grewia spp* across the regions.

Table 4: Genetic diversity within populations and genetic differentiation parameters of twelve Populations of *Grewia spp* detected by RAPD primers

Population	N	Na	Ne	I	He	P (%)
Abuhraz	**12.000**	**1.**899	**1.63**	**0.531**	**0.35**1	**91.60**
Alain	**12.000**	**1.52**1	**1.4**	**0.33**0	**0.2**26	**57.98**
ALdamazin	**12.000**	**1.52**1	**1.38**	**0.3**18	**0.2**16	**57.14**
Almnzfa	**12.000**	**1.8**57	**1.59**	**0.4**87	**0.3**82	**86.55**
Elobaid1	**12.000**	**1.58**0	**1.41**	**0.35**8	**0.2**38	**63.03**
Elobaid2	**12.000**	**1.31**1	**1.26**	**0.23**3	**0.1**55	**44.54**
Elobaid3	**12.000**	**1.5**29	**1.36**	**0.31**2	**0.21**0	**57.98**
Garsilla	**12.000**	**1.**487	**1.4**	**0.33**1	**0.2**25	**58.82**
Khartoum	**12.000**	1.**462**	**1.37**	**0.3**09	**0.21**1	**53.78**
Khoralbyed	**12.000**	**1.51**3	**1.39**	**0.3**25	**0.22**3	**56.30**
Shambat	**12.000**	**1.3**36	**1.33**	**0.27**0	**0.1**85	**46.22**
Zalingei	**12.000**	**1.47**1	**1.34**	**0.2**87	**0.19**4	**53.78**
Total	**Mean 12.000**	**1.540**	**1.405**	**0.340**	**0.234**	**60.64**
	SE 0.000	**0.02**	**0.01**	**0.01**	**0.01**	**4.12**

N_a, numberof different alleles; Ne, number of effective alleles; I, Shannon's Information Index, P (%); percentage of polymorphic bands, He; Expected Heterozygosity, SE; standard error.

Table 5: Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) of the eleven populations of *Grewia spp* genotypes.

populations	Abuhraz	Alain	Aldmazin	Almnzfa	Elobaid1	Elobaid2	Elobaid3	Garsilla	khartoum	khoralbyed	shambat	zalingei
Abuhraz	**0.000**	0.832	0.836	0.800	0.799	0.817	0.856	0.797	0.904	0.823	0.863	0.789
Alain	0.236	**0.000**	0.869	0.908	0.830	0.846	0.824	0.822	0.823	0.788	0.825	0.787
ALdamazin	0.239	0.148	**0.000**	0.817	0.811	0.878	0.823	0.837	0.823	0.860	0.802	0.934
Almnzfa	0.069	0.192	0.195	**0.000**	0.910	0.841	0.909	0.772	0.866	0.903	0.859	0.826
Elobaid1	0.191	0.221	0.239	0.100	**0.000**	0.833	0.842	0.814	0.794	0.848	0.924	0.766
Elobaid2	0.266	0.152	0.150	0.194	0.227	**0.000**	0.892	0.767	0.856	0.907	0.807	0.781
Elobaid3	0.248	0.079	0.102	0.195	0.195	0.156	**0.000**	0.812	0.769	0.867	0.805	0.823
Garsilla	0.195	0.215	0.164	0.144	0.178	0.193	0.202	**0.000**	0.847	0.883	0.914	0.744
Khartoum	0.296	0.217	0.097	0.231	0.259	0.195	0.167	0.224	**0.000**	0.869	0.812	0.815
Khoralbyed	0.204	0.090	0.142	0.155	0.206	0.096	0.130	0.187	0.223	**0.000**	0.856	0.720
Shambat	0.328	0.208	0.125	0.263	0.266	0.171	0.173	0.210	0.097	0.179	**0.000**	0.786
Zalingei	0.240	0.156	0.140	0.166	0.209	0.115	0.182	0.095	0.202	0.140	0.184	**0.000**

Principal Coordinate Analysis (PCO) and AMOVA.

Principal coordinate analysis (PCoA) available in GenAlEx v 6.501 software [23] was performed on genetic distance-based matrix on the complete data set of 144 unique *Grewia* genotypes, and on specific groups of *Grewia* genotypes defined by their original area of distribution. PCoA plots show graphically the relationship between individuals, groups of *Grewia* and determine whether partitioning into these groups is supported by genetic variation.

Principal coordinate analysis of the 144 *Grewia* genotypes generated a total variation of 10.13%, the first and the second principal coordinates explained 7.29 and 6.41of genetic variation, respectively (Figure 5).Only a few individual genotypes were separated from the others, such as, (Elobaid3.7, Aldmazin3.4, Elobaid2.8, Khartoum 11, and Khartoum 12). Although Khartoum State faraway from Elobaid3 and Aldmazin, these three genotypes were separated together may be because the Khartoum genotypes were brought by human to Elobaid3.PCoA diagram (Figure 5) showed individual accessions of Alain population from Northern Kordofan gathering together with genotypes from (Khoralbyed) samples (5,6,7,8,9,10,11) were joined to samples from (Elobaid2) and are genetically closer. Genotypes from Aldmazin were joined samples from (Elobaid2, Zalingei, Khartoum, Garsilla). PCoA diagram (Figure 5) showed genotypes (Garsilla1, Garsilla2) are genetically closer and separated from main population. Principal coordinate analysis of the12 populations showed a total variation of 45.66%, the first and the second principal coordinates explained 18.94 and 14.39 of genetic variation, respectively (Figure 6).

The RAPD data AMOVA test conducted to examine the differences among and within geographical populations was found to be statistically significant ($p < 001$) (Table 6). The test showed that 70% of the genetic variation came from within population whereas the variation due to geographic populations was 29%. The calculated PhiPT (0.287) was significant $P < 0.001$, indicating low genetic differentiation among populations. The P values were calculated for a random permutation test of 9999 permutations (Table 6).

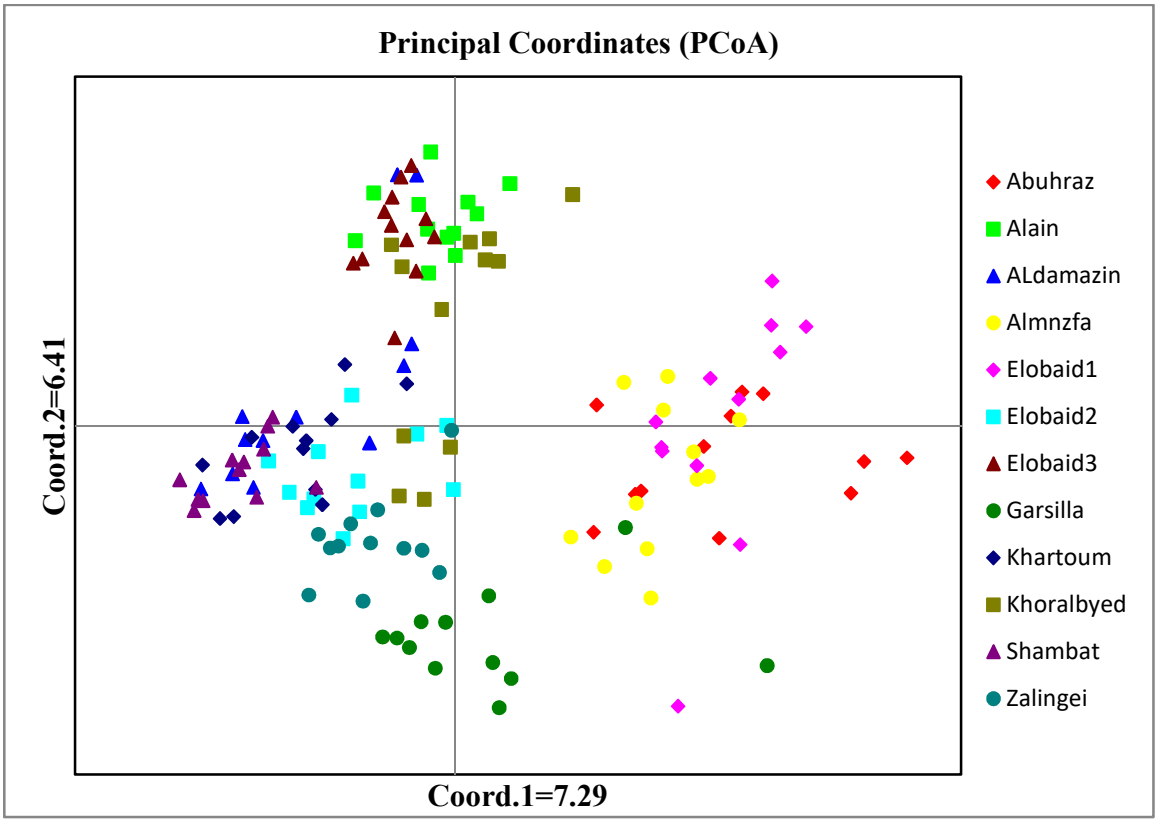

Figure 5: Principal coordinate analysis of 144 *Grewia* genotypes in Sudan based on RAPD data. The first two principal coordinates explained 7.29 and 6.41 % of the variance, respectively.

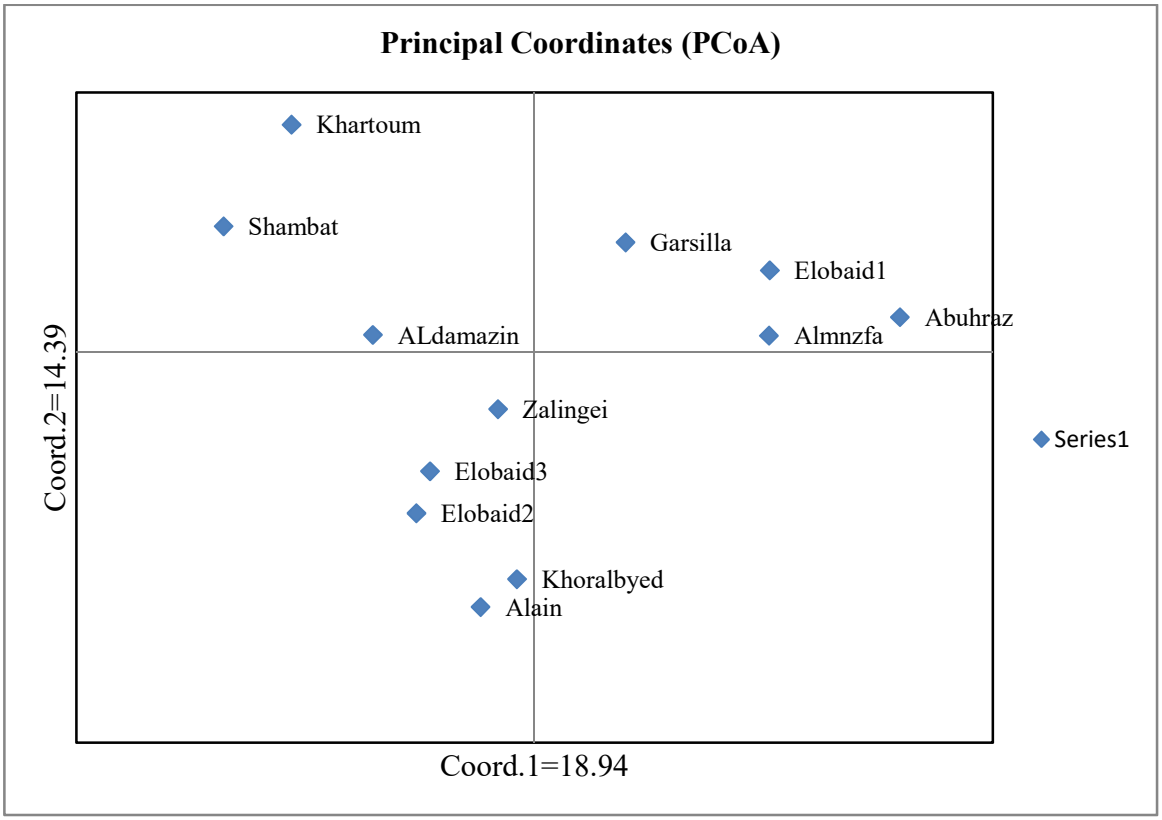

Figure 6: Principal coordinate analysis of 12 *Grewia* genotypes populations in Sudan based on RAPD data. The first two principal coordinates explained 18.94 and 14.39 % of the variance, respectively

Table 6:Analysis of molecular variance (AMOVA) within and among the populations of *Grewia spp* genotypes based on RAPD loci.

Source	Df	SS	MS	Est. Var.	% of total
Among Populations	11	846.847	76.986	5.315	29%
Within Populations	132	1743.000	13.205	13.205	71%
Total	143	2589.847	90.191	18.520	100%

Note: Df: degrees of freedom, MS=mean square, SS= sum of square, Est. Var = estimated variance.

Total Band Patterns

The highest total number of bands among the 12 studied populations was produced by *Grewia spp* genotypes from Almnzfa region producing 118 bands, followed by Abuhraz which produced 117 bands. The lowest number of bands were found in Shambat region where produced 104 bands. No private bands were found between 12 populations studied.

The highest number of locally bands found was 25% or less, Almnzfa, Alain and Elobaid3 regions were the highest ones. The almnzfa region had the highest number of locally common bands found in 50% or less with 4 bands followed by Abuhraz, Elobaid3 and Garssila. The expected heterozygosity (He) mean was highest in the Abuhraz region 0.351 and lowest in Elobaid2 region with 0.155 (Figure 7).

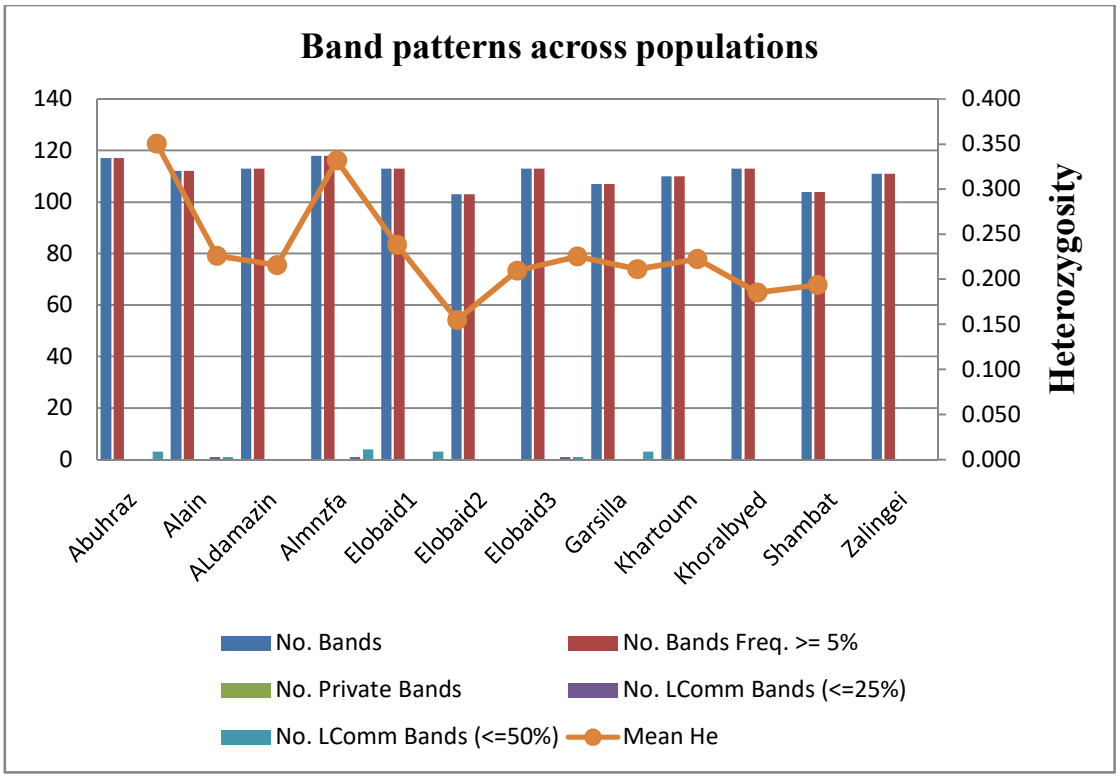

Figure 7: Total Band Patterns for binary (diploid) data by regions.

RAPD Data UPGMA Dendrogram

The tree diagram of RAPD marker analysis (Figure 4.71) showed four main clusters, cluster (A) had two groups, group I included *Grewia spp* genotypes (39, 40, 44, 41, 42) from North Kordofan, (Garsilla). Group II contained three subgroups, the first from North Kordofan and Northern west of Nyalla (43, 45, 46, 47, 48, 49, 50), the second contained (51, 53, 54, 55, 56, 57, 58, 52), from Northern west of Nyalla, the third contained (59, 60), from Northern west of Nyalla. Cluster B had three groups, group 1 contained five subgroups, the first from Northern Kordofan (Elobaid2), (61, 62, 64, 65, 63, 66, 67, 71, 68, 69, 70), the second from Northern Kordofan,(Khoralbyed),(72, 73, 74, 75, 76), the third from Blue Nile,(Aldamazin),(111, 112, 113), the fourth from Northern Kordofan (Khoralbyed), (77, 78, 79, 80), the fifth from Northern Kordofan,(Alain) (81, 82, 83, 87, 88, 90, 89, 86, 84,85). Group 2 contained five subgroups, the first from Northern Kordofan (Alain), (91, 92, 94, 95), the second from Northern Kordofan, (Alain, Elobaid3, Aldamazin), (93, 108, 104, 110, 105, 109), the third from Northern Kordofan, (Elobaid3), (98, 99, 100, 101, 102, 107), the fourth from Northern Kordofan, (Elobaid3),(96,97), the fifth from Northern Kordofan, (Elobaid3), (103,106).

Group 3 contained five subgroups, the first from Blue Nile (Aldamazin), (114, 117, 118, 122, 127, 119, 120), the second from Blue Nile (115, 116, 121, 124), the third from Southern Khartoum, (134, 137, 138, 139, 141, 142, 140, 144, 143, 136), the fourth from southern Khartoum, (123, 125, 126, 128), the fifth from Khartoum and shambat, (129,130, 133, 132, 131).

Group 4 contained three subgroups, the first and second were from Northern Kordofan (Abuhraz and Almnzfa), (2, 5, 16, 7, 8, 14, 12, 17, 18, 15, 10, 13, 9, 35, 36, 37, 38, 3, 4, 6, 11), the third from Northern Kordofan (Abuhraz, Almnzfa and Elobaid1), (3, 4, 6, 11, 21, 22, 34, 23, 24, 33, 25, 27, 26, 28, 29, 30, 32, 31).

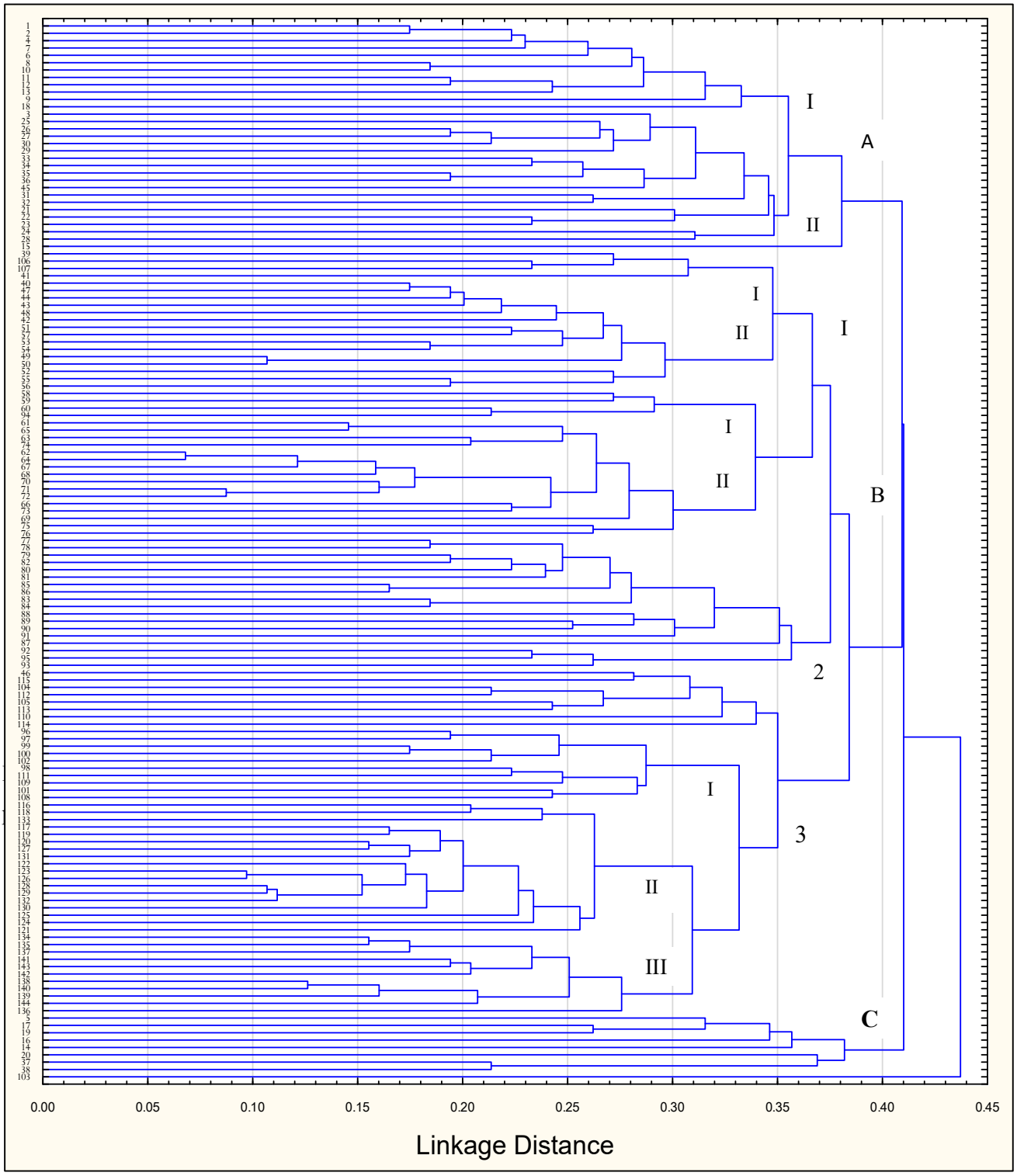

Figure 8: Dendrogram based on UPGMA analysis among 144 *Grewia spp* genotypes using 14 RAPD primers

Molecular markers have emerged as convenient methods for quantifying of genetic diversity in populations [28]. History of populations is often inferred from the variation at genetic markers that are assumed to be neutral. However, if a marker is actually subject to selection, conclusion based on patterns of genetic variation could be misleading [29, 30]. Since neutral marker alleles could be linked to deleterious mutations or selectively favored alleles genetic variation can erode faster than expected under neutral assumptions [31]. Study of genetic diversity is very important because it can give a clear picture of whether a species can survive in the long run or not. A population with low genetic diversity cannot tolerate negative environmental impacts as most of the population is identical [32]. But population with higher genetic diversity can lead to individuals with a new genetic makeup which may make them survive under adverse conditions [33].

The genetic diversity studies using molecular markers have become useful due to their better reliability and high resolution.

The genetic dissimilarity values obtained with RAPD have been introduced for measuring genetic relationships in many plant species for easiness of the method, which only requires PCR technology. The low reproducibility of RAPD [34], introduces problem when used for cultivar identification compared with the other marker applications. In the present study, however, it was found that once the PCR conditions were well setup, high reproducibility for the RAPD markers was observed. Apart from surveying different genomic regions, the marker distribution throughout the genome and the coverage of DNA targets of each specific marker assay provide additional information [35]. Therefore, arbitrary (RAPD) markers were used in the present study.

Very limited work has been carried out to study the diversity of *Grewia tenax* at molecular level. Only two such reports are available in *Grewia spp* genotypes [36,37].The present study is the first report evaluating the genetic diversity of large number of *Grewia tenax* samples in Sudanutilizing multiple marker systems which are easy to handle and amenable for PCR-based analysis. Since no reports of RAPD analysis are available in Sudan, therefore, we have discussed the results in comparison to other tree species.

In the present study, 14 of RAPD markers (Table 3) were utilized to assess the genetic diversity of 144 *Grewia tenax* samples belonging to 12 different populations collected from 12 districts of In this study the

mean level of polymorphism revealed by RAPD is higher (94%).

Population genetics in varieties of *Grewia tenax* were studied using RAPD markers. In present study, the applicability of RAPD is compared as genetic marker to characterize the population of *Grewia tenax.* The results indicate that percentage of RAPD polymorphic bands are higher (94%), these findings are in disagreement with [37] who reported screening twenty-five RAPD primers in *Grewia optiva*, reporting 96.31%.

The genetic diversity among the samples of 12 populations of *Grewia spp* was also dissected using Nei's genetic diversity (He) and Shannon's information index (I) which gives an indication of the pattern of evolutionary forces such as mutation/selection/migration and the reproductive method of the organism [38]. The average 'He' and 'I' values obtained for RAPD and markers were 0.34and 0.406 respectively (Table 4).

The AMOVA of the 12 populations revealed that higher genetic variations existed within the populations i.e., 77%, 29 % with RAPD marker systems respectively. The dendrograms based on RAPD markers showed partially different genetic distance levels than when used individually. But when used together, RAPD-based cluster is more similar to the combined cluster than ISSR-based cluster. Results are in agreement with the studies in *Grewia optiva* species [37]. However, in Lupin (*Lupinus* sp.) it was found that ISSR-based cluster is more similar to the combined cluster than RAPD-based cluster [39]. *Jatropha curcas* also showed similar result when RAPD and ISSR dendrogram patterns were combined [40].Cluster analysis was carried out on marker profiling data based on RAPD. The results based on all the DNA marker profiles broadly grouped the 12 populations into three clusters. There was close relationship between some of the populations used in this study; presumably they might have been collected from similar locations.

The result of this study showed Some private bands were detected within genotypes (Table 4). These could be useful for further study by designing special marker for the discriminating the genotypes. From this study, it can be concluded that there is significant variation among 144 *Grewia spp* genotypes collected from different locations of Sudan on the basis of RAPD and ISSR data. This study provides us with good knowledge about genetic variability of *Grewia tenax* which may allow more efficient and effective use of resources in plant improvement programs.

CONCLUSIONS

It was concluded that the marker systems RAPD can be effectively used in determination of genetic relationships among *Grewia tenax* genotypes.

The mean genetic diversity among the *Grewia spp* genotypes within the States is higher than that among States of Sudan.This can be attributed to the open pollinated nature of the species as well as the human activity in agriculture where farmers move the *Grewia* seeds from one place to another.

Grewia spp genotypes from the Abuhraz state had the highest number of bands; number of private bands; percentage of polymorphic loci and heterozygosity mean (*He*) than accessions from other states.

In our study the ground distance between the collection sites does not correlate well with the genetic distance due to the gene flow. This gene flow created a great variability within populations as detected by the RAPD, AMOVA analysis proved that and also showed that most of the genetic diversity was due to within States rather than among States variations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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