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Full Length Research Paper

Genetic variation within and among five natural populations of endangered *Sclerocarya birrea* (A. Rich) subsp. *Birrea* in Sudan

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Knowledge of genetic diversity is important for successful conservation and domestication of species. In order to determine genetic diversity within and among *Sclerocarya birrea* (A. Rich.) Htochst. subsp. *birrea* populations in Sudan, random amplified polymorphic DNA (RAPD) markers were used. Leaf materials from 75 seedlings from five populations (Rashad, Alfaid, Alkhwi, Aldamazin and Baw) of this species were used to compare the genetic diversity. A total of 37 bands were generated using four primers. Genetic variation within the populations as estimated by Shannon information index ranged from 0.343 to 0.272 with an overall diversity of 0.306. Analysis of molecular variance revealed that 46% of the variation was attributed to differences among the populations and 54% within the populations (P < 0.001). The gene flow among population was small (N*m* = 0.297). UPGMA cluster and principal coordinate analyses (PCA) indicated Alfaid as the most distinct population. Since genetic variation was found to occur among and within the populations of *S. birrea* (A. Rich.) Hochst. Subsp. *birrea*, conservation of these populations would help in the maintenance of the species.

Key words: *Sclerocarya birrea* subsp. *birrea*, genetic variation, conservation, random amplified polymorphic DNA (RAPD), Sudan.

INTRODUCTION

Sclerocarya birrea (Anacardiaceae) is a common and widespread species throughout the semi-arid, deciduous savannas of much of sub-Saharan Africa (Peters, 1988). It is common in wooded grasslands, riverine woodland and bush land, being frequently associated with rocky hills. It is widely used by rural populations in most countries in which it is found for its fruits, timber, bark and even its roots (Palmer and Pitman, 1972; Shone, 1979; Walker, 1989; Shackleton et al., 2000). *S. birrea* consist of three subspecies (*S. birrea* subsp. *birrea*, *S. birrea* subsp. *caffra* and *S. birrea* subsp. *multifoliolata*) distributed in about 29 African countries from southern Africa through east Africa to west African and Madagascar (Fox and Norwood-Young, 1982; Arnold and De Wet, 1993) and Tanzania is a centre of diversity for *S. birrea* with the three subspecies (Akinnifesi et al., 2006). Muok et al. (2000) reported that the tree is appropriate for introduction into dry land agroforestry systems and is highly valued by local communities for its potential for domestication and commercialization in Africa have been gaining international attention (Hall et al., 2002).

S. birrea (A. Rich.) Hochst. subsp. *birrea* is one of the natural tree species in Sudan, it occurs in low rainfall woodland savannah and is a constituent of low elevation (mostly < 1600 m) vegetation, subsp. *birrea* can occur at around 1700 m at Jebel Marra area (Miehe, 1988). The species is under threat from extensive loss and fragmentation due to unregulated cutting for timber and fuel wood, clearing of forestland for agriculture, overgrazing of

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livestock, burning and civil wars (Gorashi, 2001) rendered the species vulnerable to loss of natural regeneration, reduced population sizes calling for urgent conservation and management of remnants populations.

For conservation and use of a species, a detailed knowledge of the genetic variation within and among populations is required (Dawson et al., 1995). The preservation of genetic diversity has become a common objective of most conservation programmes (Burgman et al., 1993; Geburek, 1997) and defining genetic diversity within and among natural populations is a necessary first step to achieve this goal (Holsinger and Gottlieb, 1991).

In the last two decades, the use of molecular markers to study the genetic diversity in natural, managed and cultivated stands of populations and species at DNA and protein levels, has increased greatly for identifying the genotypes, assessing and exploiting the genetic variability (Whitkus et al., 1994) and for understanding the consequences of historical events, such as range expansion, fragmentation and bottleneck for the distribution of populations (Mortiz, 1995).

The wide use of molecular markers in analyzing the pattern of variation within and among natural populations was reviewed by Newton et al. (1999). Genetic markers have been developed to analyze and estimate genetic diversity, but no single technique is universally ideal; each available technique has both strengths and weaknesses (Mueller and Wolfenbarger, 1999). Among the various markers, random amplified polymorphic DNA (RAPD) (Williams et al., 1990) is one of the most popular DNA-based approaches (Martin and Hennandez, 2000; Bekessy et al., 2002). Furthermore, RAPD is the least technically demanding marker and offer a fast method for providing information from a large number of loci, particularly where no studies has previously been undertaken. Moreover, the diversity assessed by RAPD is comparable with that obtained with other techniques such as isoenzymes (Hamrick and Godt, 1990) or restriction fragment length polymorphism (RFLP), (Wu et al., 1999). Advantages of RAPD include suitability for work on anonymous genomes, applicably to work where limited DNA is available, efficiency and low expense (Hadrys et al., 1992). Some limitations of RAPD exist, however, owing to their lack of reproducibility and identical pattern produced by homozygous and heterozygous individuals (Harris, 1999).

The technical ease of RAPD markers and their application to any species has led to their use in many studies in forest trees, both in genetic linkage mapping and population genetics (Young et al., 2000). RAPD technique has been successfully used to study genetic diversity in many forest tree species (Nesbitt et al., 1995; Rajagopal et al., 2000; Newton et al., 2002; Pither et al., 2003; Eren et al., 2004; Rashmi et al., 2004; Kim et al., 2005; Jamnadass et al., 2005; Chiveu et al., 2008; Hamza, 2010).

The available information on molecular genetic variation in *S. birrea* was focused on southern part of its ranges mainly in subsp. *Caffra* and *multifolilata* according to the studies of Agufa (2002), Kadu et al. (2006) and Muok et al. (2007). In these studies, RAPD analysis enabled a cluster analysis based on genetic distance to summarize relations among 16 populations and estimates of within population diversity. Five additional Kenyan populations of *S. birrea*, not identified to subspecies, were included by Muok (2000), in supplementary RAPD analysis to that of Agufa (2002). Muok found little evidence of groupings among the populations, but the Kenyan populations were more closely related to each other than to Malawi or Mali population. The analysis of Gutman et al. (1999) confirmed the presence of genetic differences between clones but did not indicate consistent differences between populations of Botswanan and South African origin.

The objective of this study was to quantify the genetic variation of *S. birrea* subsp. *birrea* in order to provide baseline data on genetic variations among and within the endangered populations of the species in Sudan using RAPD technique.

MATERIALS AND METHODS

Sample collection

Five populations of *S. birrea* subsp. *birrea* namely: Baw, Alkhwi, Aldamazin, Rashad and Alfaid were studied (Table 1; Figure 1). A total of 75 individuals from the five populations were sampled (15 individuals per population). A minimum distance of 100 m was maintained between mother trees for maximizing genetic variation within populations. For each individual, fresh leaf samples were collected for DNA extraction.

DNA extraction

Genomic DNA was extracted from fresh leaf tissue of the seventy five (75) plants using modified CTAB (cetyltrimethyl ammonium bromide) method (Doyle and Doyle, 1990). Two grams of the young leaf tissue of the seventy five (75) samples were ground using dry ice and blender, the fine powder of each sample was then transferred into 13 ml Falcon tube containing 6 ml preheated (60°C) CTAB buffer. The solution was incubated in water bath at 60°C for 30 min and inverted periodically. The tubes were left to cool at room temperature, 3 ml of chloroform isoamyalcohol (24:1) were added to each tube and the solutions were mixed gently and thoroughly for 10 min then centrifuged at 4000 rpm for 15 min. The aqueous phase was transferred by pipette into a new tube and the precipitates were discarded. The addition of equal volumes of chloroform isoamy-alcohol (24:1) and the discarding of the precipitates were repeated twice. Equal volume of cold 2-propanol was added to each tube and mixed gently to precipitate the DNA in freezer for 30 min. The cool solutions were centrifuged at 4000 rpm for 15 min, then the supernatants were discarded and equal volumes of 70% ethanol were added and then centrifuged at 4000 rpm for 5 min. The addition of 70% ethanol was repeated once. The supernatants were discarded to allow the formed pellet to dry for 30 min at room temperature. The dried DNA pellet was resuspended in 200 to 300 µl of TE buffer (10mM Tris, 1 mM EDTA (PH 8) and stored at -20°C.

The extracted DNA samples were quantified using gel electrophoresis. The gel was prepared by dissolving 1 g of agarose

Table 1. Geographical locations of studied *S. birrea* subsp.*birrea* populations in Sudan.

Population name	Population code	Location	Latitude (N)	Longitude (E)	Altitude (m)
Baw	BA	Blue Nile	11°20′ 24″	34°05′ 04″	481
Alkhwi	KW	Northern Kordofan	13°6′ 37″	29°07′33″	685
Aldamazin	DZ	Blue Nile	11°50′ 50″	34°29′22″	493
Rashad	RD	Southern Kordofan	11°49′ 28″	31°02′50″	840
Alfaid	FD	Southern Kordofan	11°7′ 33″	30°29′ 22″	615

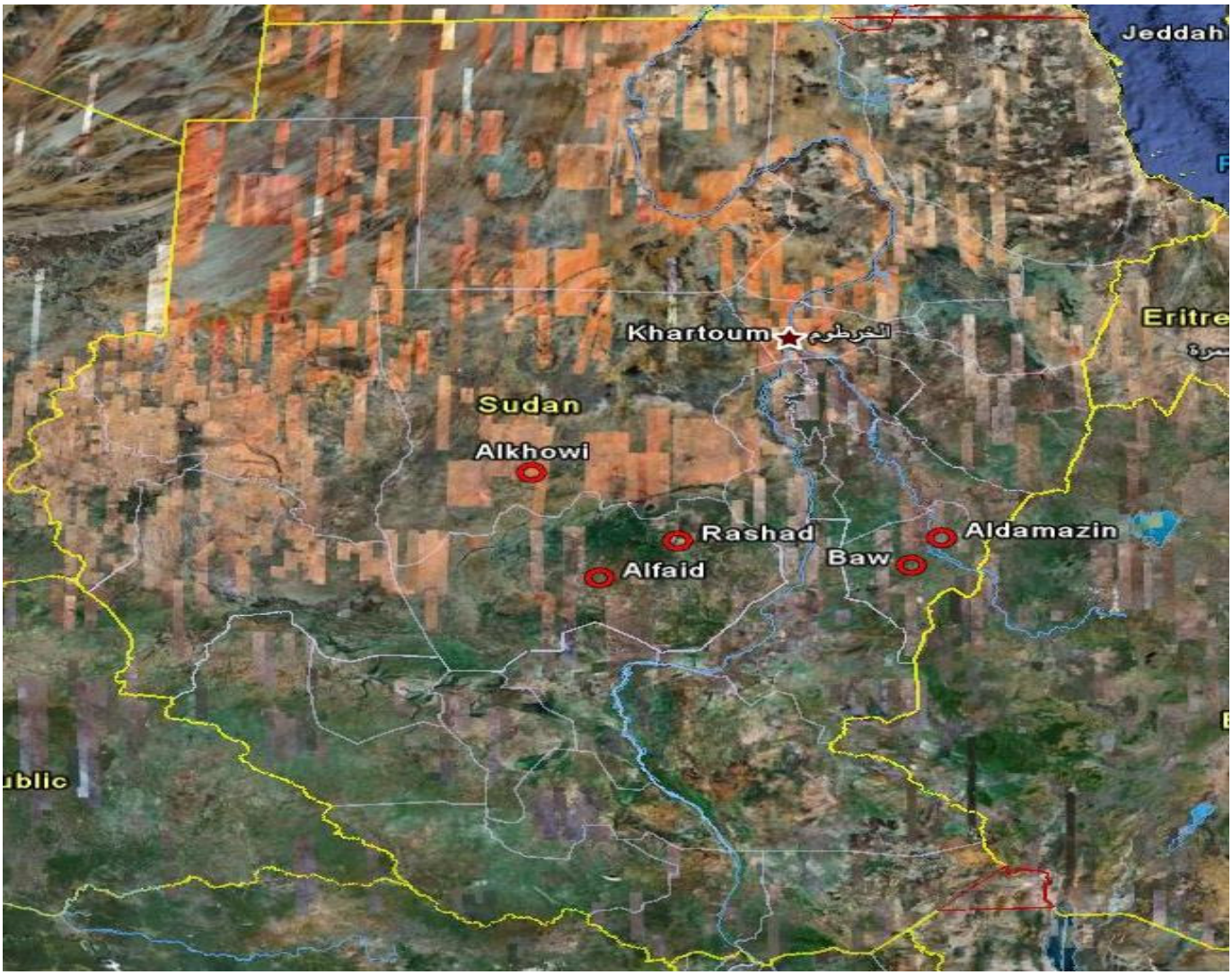

Figure 1. Map of Sudan showing the locations of the five studied forests.

in 100 ml 1xTBE (Tris-borate EDTA), the solution was heated in microwave oven for 2 min until it became clear and warm, 1.5 µl of ethidium bromide were added. The gel was poured into the gel tray and then the combs were placed to make wells after the gel cools down. 3 µl of the DNA mixed with 2 µl loading dye were loaded into the 1% agarose gel. The gel was submersed in 1xTBE buffer and run at 60 V. The DNA quantification was performed visually under UV and photographed. The resuspended DNA samples were then diluted in sterile distilled water for use in polymerase chain reaction.

Random amplification polymorphic DNA (RAPD) analysis

For standardization of the amplification, a total of ten random decamer oligonucleotide primers corresponding to kit A and B from Operon Technologies (Alameda, California, USA) and UBC, Canada were: OPA03 (5′-AGTCAGCCAC-3′), OPA09 (5′-GGGTAACGCC-3′), OPA17 (5′- GACCGCTTGT-3′), OPA20(5′-GTTGCGATCC-3′), OPB08 (5′-GCGGCTGGAG-3′), OPB20 (5′-GGACCCTTAC-3′), UBC101 (5′-GTCCACACGG-3′), UBC104 (5′-GGGCAATGAT-3′),

UBC122 (5'-GTAGACGAGC-3'), and UBC155 (5'-CTGGCGGCTG-3').

The primers were initially tested using the five samples (one sample per population), to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products. To ensure reproducibility, the primers generating no or weak amplification were discarded.

Polymerase chain reaction (PCR) and electrophoresis

The PCR reactions were carried out in 25 µl volume containing 15 µl sterile distilled water, 2.5 µl 10X*taq* buffer, 2.5 µl (2 mM/µl) dNTPs,1.5 µl (50 mM) $MgCl_2$, 2 µl (10 pmol/µl) primer, 0.5 µl(5u/ µl) *Taq* DNA polymerase and 1 µl (10 to 30 ng/µl) template DNA. Amplification was achieved in a Biometra thermocycler (T.Personal 48, Germany), programmed as initial denaturation at 94°C for 5 min, then 40 cycles each start with denaturation at 94°C for 1 min followed by annealing 36°C for 1 min and ended by extension at 72°C for 7 min. Amplification products were separated alongside a molecular weight marker (1kb ladder, Cinagen Co.) in 1.5% agarose gel containing 1.5 µl ethidium bromide using 1XTBE buffer at 60 V. The gel was visualized and photographed using Gel Doc System (Gel Doc. 2000, BioRad, USA).

Statistical analyses

Each PCR product was assumed to represent a single locus and was scored into 0 or 1. For each genotype the presence of a band (1) or its absence (0) was scored manually and a binary matrix was generated for analysis using the program *GenALEX* ver. 6 (Peakall and Smouse, 2006).

Genetic diversity

For each population the number of observed alleles (Na), number of effective alleles (Ne), percentage of polymorphic loci (%P), the expected heterozygosity (He) and genetic variation within the studied populations were estimated in term of Shannon's information index (I) (Lewontin, 1972). The estimates of Nei's (1972), standard genetic distance for all pair-wise populations were calculated. Genetic variation was also evaluated by analysis of molecular variance (AMOVA) (Excoffier et al., 1992) the significance of the variance components was tested by calculating their probabilities, based on 1000 iterations.

Indirect gene flow (N*m*) estimation was obtained from PhiPT (analogue of F_{ST} fixation index) values according to Wright's equation: N*m*= [1-FST]/4FST (Wright, 1951).

Genetic structure

To examine the genetic relationship within and among the populations, a cluster analysis was performed by unweighted pair group method arithmetic average (UPGMA), using Statistica software ver. 9. A dendrogram was constructed based on the cluster analysis. Principal coordinate analysis was done using the program *GenALEX* ver.6. In addition, the geographic distances among populations were estimated from coordinates obtained using a geographical positioning system. The relationship between the genetic distance matrix and the geographic distance matrix was analyzed using a Mantel type matrix randomization test (Mantel, 1967). In this analysis, distances from one matrix are regressed onto the distances in the other matrix being tested. If the observed regression exceeded 95% of the randomly generated regressions, the observed regression was considered statistically significant.

RESULTS

Genetic diversity

Considering the five populations, a total of 37 reproducible bands were found with four primers out of the ten primers tested. The total number of bands generated per primer ranged between 7 (primer OPA20) to 11 (primer OPB08) bands. The percentage of polymorphic bands ranged from 90 to100%.

The population with the highest polymorphic loci was Rashad (62.16%), followed by Alfaid (59%). The other three populations (Alkhwi, Aldamazin and Baw) had the same percentage of polymorphism (56.76%). The overall mean of population's polymorphic loci (%P) was 58.38%. Based on Shannon's information index, Alkhwi population had the most genetic diverse (0.343) with the highest number of effective alleles (1.429), while (Aldamazin) population was the least diverse population (0.272) and the least number of effective alleles (1.307). The expected heterozygosity (He) varied from 0.237 (Alkhwi) to 0.180 (Aldamazin) (Table 2 and 3).

The Nei's genetic distance between pairs of populations varied from 0.381 between Alfaid and Aldamazin populations to 0.198 between Alkhwi and Baw populations. Thus, the most genetically distant populations were Alfaid and Aldamazin (Table 4). In addition, no significant correlation was found between pairwise genetic distance and geographic distance in regression test (R^2= 0.0357, $P < 0.05$), suggesting that geographic distance had no influence on genetic variation (Figure 2).

Estimated gene flow for the studied populations was N*m* = 0.297, indicating that the genetic exchange bet-ween populations is very low. The partitioning of genetic variation was examined by the analysis of molecular variance (AMOVA). The five populations showed significant differentiation ($P < 0.001$), with 54% of the differentiation attributed to within populations variation and 46% attributed to among populations variation (Table 5).

Structuring of genetic diversity

The first three principal coordinates derived from principal coordinate analysis (PCoA) based on covariance matrix for the sampled individuals of the five populations described 28.79, 21.75 and 19.26% of the total variance. Plots of the first two coordinates are shown in Figure 3. The plots of four populations Rashad, Alkhwi, Baw and Aldamazin were closer to one another or overlapped, whereas Alfaid population was clearly separated.

The genetic relationships among the five populations were summarized using UPGMA cluster analysis (Figure 4). The dendrogram contained of two clusters and one out group. The first cluster grouped all the individuals of Rashad and most Baw populations, whereas the second cluster contained Aldamazin and Alkhwi populations.

Table 2. Polymorphism detected by the use of four random primers on five populations of *S. birrea* subsp. *birrea.*

Primer name	Sequence (5'- 3')	Total number of band	Number of polymorphic band	%of polymorphic band
OPA20	GTTGCGATCC	7	7	100
UBC104	GGGCAATGAT	10	10	100
OPB08	GTCCACACGG	11	10	90
OPB20	GGACCCTTAC	9	9	100

Table 3. Number of different alleles (Na), number of effective alleles (Ne), Shannon index (I), percentage of polymorphic loci (%P) and the expected heterozygosity (He) of the five *S. birrea* subsp.*birrea* populations.

POP	Na	Ne	I	%P	He
BA	0 .486±0.107	0.321±0.059	0.289±0.047	56.76%	0.192±0.032
KW	0 .432±0.120	1.429±0.069	0.343±0.052	56.76%	0.237±0.037
DZ	0 .486±0.107	1.307±0.061	0.272±0.047	56.76%	0.180±0.033
RD	1.486±0.120	1.404±0.071	0.326±0.050	62.16%	0.222±0.036
FD	1.459±0.120	1.34±0.063	0.298±0.047	59.46%	0.198±0.033
Mean	1.47±0.051	1.36±0.029	0.306±0.022	58.38±0.08	0.206±0.034

Table 4. Nei's genetic distance (above diagonal) and the geographic distance in km (below diagonal) for pairwise differences between the five populations of *S. birrea* subsp. *birrea* studied in Sudan.

POP	BA	KW	DZ	RD	FD
BA	-	0.198	0.306	0.213	0.309
KW	573.78	-	0.244	0.277	0.310
DZ	73.89	600	-	0.291	0.381
RD	339.14	250.98	372.86	-	0.370
FD	445.47	242.71	494.41	135.32	-

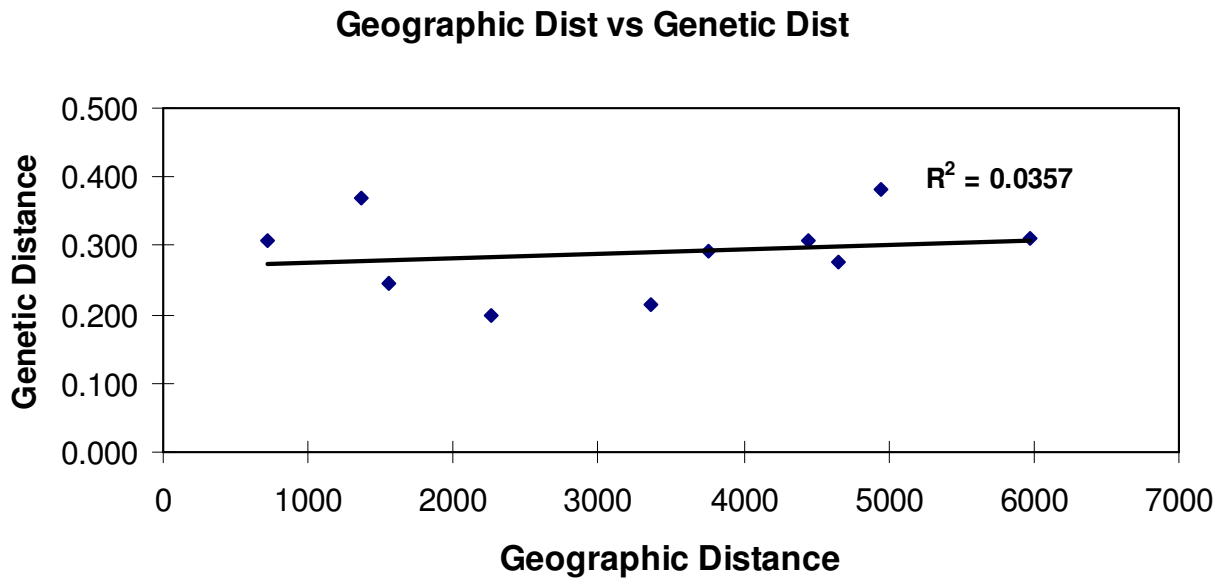

Figure 2. Regression analysis of geographic distance against genetic distance of the five populations of *S. birrea* subsp.*birrea*.

Table 5. Summary of the analysis of molecular variance (AMOVA) within and among populations of *S. birrea.*

Source	df	SS	MS	Estimated variance	%	Value	Probability
Among pops	4	204.48	51.12	3.157	46		
within pops	70	263.067	3.758	3.758	54	0.457	0.001
Total	74	467.547		6.916	100%		

The analysis is based on RAPD phenotypes consisting of 37 band states levels of significance are based on 1000 iterations.

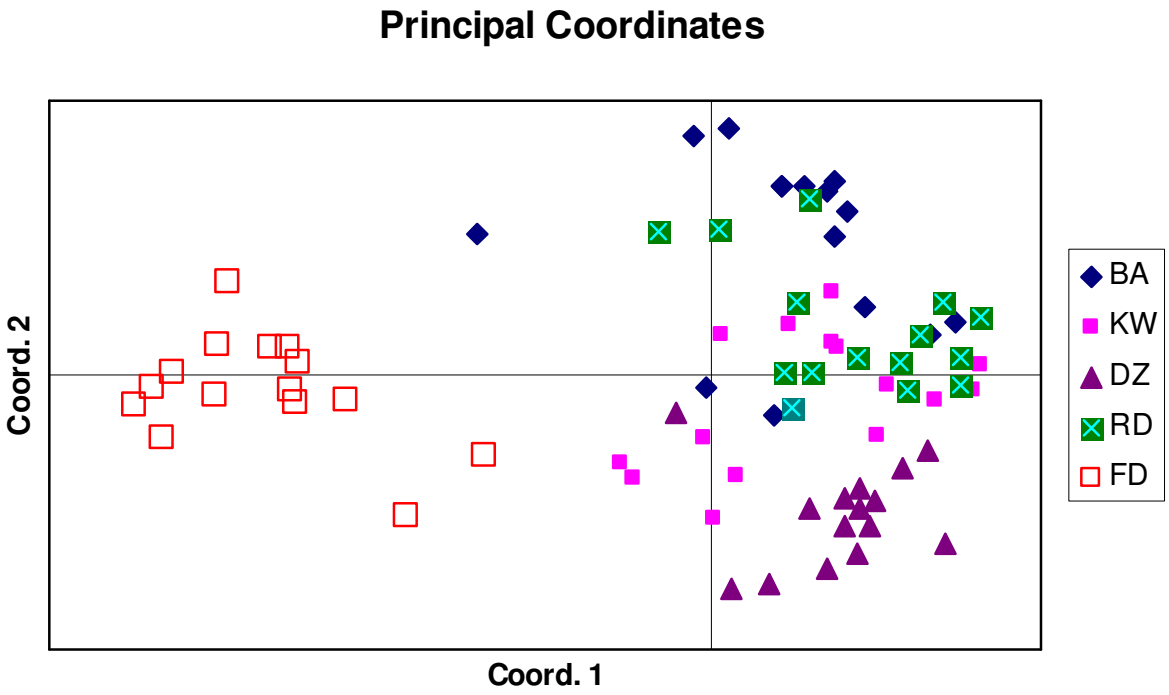

Figure 3. Plotting of the first two principal coordinates for each individual of the five *S. birrea* subsp. *birrea* populations.

Alfaid population was the most distinct from the other four populations and came as out-group. Principal coordinate analysis (PCoA) was conducted to further examine the relationships among the populations. These results sup-ported the principal coordinate's analysis.

DISCUSSION

Random amplification of polymorphic DNA (RAPD) proved to be a high informative method for the detection of genetic variation within and among populations of *S. birrea* subsp. *birrea* in Sudan. By using four primers 37 RAPD- phenotypes were differentiated out of 75 studied individuals. More primers might have resulted in an even higher number of different phenotypes. It is useful to compare the genetic variation in *S. birrea* subsp. *birrea* with other tree species with similar life history, geographic range and breeding system. In this study, the mean value of the percentage of polymorphic loci for all populations studied was 58.38%. The percentage of polymorphic loci was 78·34% in *Adansonia digitata* (Assogbadjo et al., 2006), 60.6% in *Acacia nilotica* (Khadidiatou et al., 2008) 84.4% in *Acacia senegal* (Chiveu et al., 2008) and 60.3% in *Tamarindus indica* (Diallo et al., 2007). The percentage of polymorphic loci of some endangered plant species for example *Capparis decidua* was 54.87% (AbdelMawgood et al., 2006), *Changium smyrnioides* was 69% (Fu et al., 2003) *Lactoris fernandezziana* was 24.5% (Brauner et al., 1992) and *Dacydium pierrei* was 33% (Su et al., 1999). This indicates that *S. birrea* subsp. *birrea* may fall within the range of endangered species, but the studied popu-lations still had genetic diversity that should be able to fit environmental variation. The results of percentage of polymorphic loci suggest that Rashad was the highest diverse population and Aldamazin was the least, while Alkhwi, Alfaid and Baw had similar level of genetic variation.

Alkhwi and Rashad had the highest genetic diversity in this study (0.343 and 0.326). Similar ranges were obtained

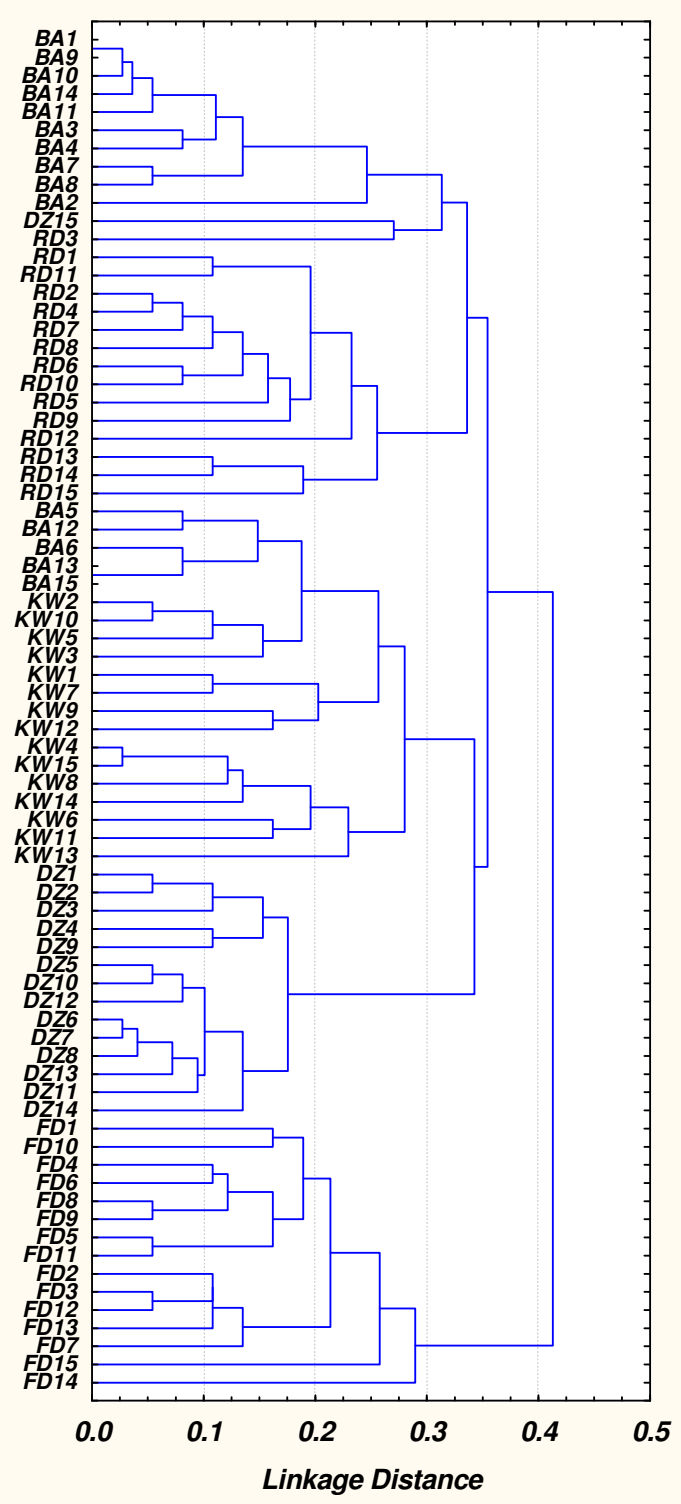

Figure 4. UPGMA tree *based* on 37 RAPD loci, showing relationships among the five *S. birrea* subsp.*birrea* populations. Numbers indicate individual within population code.

by Muok et al. (2007), studying six populations of *S. birrea* in Kenya by using the same molecular technique. In their study, the population with the highest diversity in their study had a Shannon's information index of 0.4430 followed by 0.3433. The later are the similar estimates of diversity obtained in our study for Rashad population. The population with the least diversity in this study was Aldamazin (I= 0.272), this is higher than that obtained by Muok et al. (2007), as in their study, the least genetically diverse population had less diversity (I= 0.1959). According to AMOVA, there was significant partitioning of genetic variation ($P < 0.001$) with among population value of 46% indicating genetic differentiation, was higher than 18.7% reported by Kadu et al. (2006). A higher diversity rate within population is expected for *S. birrea*, as it is a dioecious species with no or very little selfing. The within population diversity (54%) was lower than that (71.2%) which was obtained by (Kadu et al., 2006). Generally, the within population variance should be high in large population, the pattern of the extent of genetic variability within and among *S. birrea* in Sudan may be affected by reduced population sizes due to human activities. These activities may explain the substantial higher level of among populations variation as well as lower within population variation.

The Nei's genetic distance matrix between pairs of populations (Table 4) showed an average distance range from 0.381 to 0.198 with a mean of 0.289. Thus, the populations tested in the study are less divergent at the DNA level reflecting a decrease in genetic differentiation in the naturally *S. birrea* subsp. *birrea* fragmented populations in Sudan. A weak correlation between genetic distance and geographical distance (R^2= 0.0357) was observed unlike that obtained by (Kadu et al., 2006). From PCoA and cluster analyses (Figures 3 and 4) it is evident that population differentiation did not result from geographical isolation. This was demonstrated by FD population as being more distinct from all populations even from RD population which is geographically proximate. Low levels of populations' differentiation in tropical tree species were reported by Hamrick and Loveless (1989). Low gene flow (N*m* = 0.297) value indicated low level of gene exchange (pollen and seed) among *S. birrea* subsp. *birrea* populations, which is a characteristic of many plant species (Slatkin, 1985) and *S. birrea* is insect pollinated species with relatively large seed (stone) and occur at lower densities which might be expected to have less gene flow (Young et al., 2000); small herbivorous animals are also vectors for seed dispersal which will not be expected to travel long distances. According to Wright (1969), the critical value for N*m* is 0.5. N*m* value below 0.5 indicates that populations will diverge extensively as a result of drift. Overall, the calculated gene flow (N*m*) indicates the population of *S. birrea* subsp. *birrea* in Sudan in the process of differentiation due to genetic drift .In their study, Kadu et al. (2006), used RAPD technique to study 16 populations of *S. birrea* subsp. *caffra*. Their findings supported the hypothesis that *S. birrea* have a complex evolution, with contributions of both seeds and pollen to the gene flow, they reported highest chloroplast DNA haplotypes.

Genetic diversity provides a template for adaptation and evolution of populations and species; therefore, maintenance of high genetic diversity in *S. birrea* subsp. *birrea* in Sudan is one of the most important issues for conservation of this endangered species. Based on the presence of high genetic variation, Rashad population should be considered as *in situ* reserved and is valuable since it offer gene pool from which gene conservation and improvement programs can be made. The population which had lowest diversity based on Shannon index was Aldamazin (0.272), this population is small in size around 100 trees and lack adequate protection from human activities especially subjected to heavy grassing (personal observation) that resulted in absence of new regeneration despite the favorable condition for growth and seed production.

This is the first study describing the evaluation of genetic diversity in *S. birrea* subsp. *birrea* at the population level in Sudan using RAPD markers. As shown in this study, the RAPD technique was a suitable molecular technique to study the genetic diversity in *S. birrea*. The mean diversity estimates of Shannon's had shown the existence of genetic variability among populations. The pair wise comparison of genetic distances between the populations was low. The constructed dendrogram was able to discriminate the studied populations into three clusters, Alfaid population made a distinct group. To make more effective the outcome of the genotyping, the researchers have to increase the number of the sampled populations, sample size and the number of primers used in the study. More powerful molecular markers should be implemented in economically and environmentally important species.

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