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Full Length Research Paper

Phylogenetic relationships within indigenous Sudanese *Cassia senna* (L.) using RAPD molecular markers

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Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *Cassia senna* (L.). RAPD technique was carried out in a set of 27 (*C. senna* (L.) genotypes collected from different regions of Sudan. A total of 27 polymorphisms (5.2 polymorphic markers per primer) out of 31 reproducible products (7.5 fragments per primer) were obtained from the 6 primers used. The number of bands per primer ranged from 4 to 7, whereas the number of polymorphic bands ranged from 3 to 6, corresponding to 60% of the amplification products. Low level of genetic similarity was observed in the collected genotypes. Six random primers, each with 10 bases generated a total of 27 polymorphic bands out of the 31 total bands, that is, polymorphism of 60% was observed. Molecular variance (STATISTCA) was used to investigate the genetic diversity of the populations. UPGMA clustering resulted in two major clades. It was concluded that high level of polymorphism found suggests that RAPD techniques can be useful for studying *C. senna*.

Key words: *Cassia senna*, genotypes, Sudan, genetic diversity, RAPD.

INTRODUCTION

Cassia senna (L.) is widely distributed in central Sudan (Hayati, 2005). It is present in western and eastern Sudan, on the Nubian gravel desert and along the river Nile course from Khartoum to Dongola in Northern Sudan and it occurs on all types of soils with best yield on clay soils (Elamin, 1990; Vetaas, 1993).

C. senna is undoubtedly the most reputed indigenous medicinal plant of the Sudan. Pods are a rich source of pharmaceutically active anthraquinones, which is in high demand by international companies and are widely used as laxative and described in most pharmacopoeias (WHO, 1999).

Senna displays a high diversity of habits, including herbs, shrubs, treelets, tall trees and lianas and has successfully colonized a wide range of habitats in different climates and latitudes. Of the approximately 350 species currently ascribed to the genus, 80% occur on the American continent, while most of the remaining members are found in tropical Africa, Madagascar and Australia, with only few species in southeastern Asia and some on the Pacific Islands (Irwin and barneby, 1982; Randell and Barlow, 1998; Randell, 1990). No *Senna* species are native to Europe, although several of them have long been used in the European medical tradition (e.g. Colladon, 1816).

The use of DNA profiling techniques for genotype identification offers several advantages over the use of morphological data. DNA sequences are independent of environment conditions; furthermore, identification can be determined at any stage of plant growth (Smith and Smith, 1992) and very similar genotypes, including clonal variants, can be discriminated using DNA techniques (Demeke et al., 1992). The use of polymerase chain reaction (PCR) has enabled the development of simple and rapid DNA profiling methods (Morell et al., 1995). Molecular markers are best tools for determining genetic relationships. With the development of PCR based RAPD (random amplified polymorphic DNA) and SSR (microsatellites); most of problems associated with RFLP (restriction fragment length polymorphism) were overcome. RAPD markers offer quick screening of different regions of the genome for genetic polymorphisms. The technique of RAPD gained impor-

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Table 1. List of *C. senna* genotypes studied and their area and region of collection.

Region	Area	Genotype
Central Sudan	Algazira	S1
Central Sudan	Algazira	S2
Central Sudan	Algazira	S3
Central Sudan	Algazira	S4
Central Sudan	Algazira	S5
Central Sudan	Algazira	S6
Central Sudan	Algazira	S7
Central Sudan	Algazira	S8
Central Sudan	Algazira	S9
Central Sudan	Algazira	S10
Central Sudan	Algazira	S11
Central Sudan	Algazira	S12
Central Sudan	Algazira	S13
Omdurman	Alwadi street	S14
Omdurman	Alshati street	S15
Omdurman	Aboangaa 1	S16
Omdurman	Aboangaa2	S17
Omdurman	Aboangaa3	S18
Northern Khartoum	University of Khartoum Agric	S19
Northern Khartoum	Northern Bahry	S20
Northern Bahry	Nabta 1	S21
Northern Bahry	Samrab	S22
Northern Bahry	Khartoum university	S23
Northern Bahry	Nabta 2	S24
Eastern Sudan	Port Sudan 1	S25
Eastern Sudan	Port Sudan 2	S26
Eastern Sudan	Port Sudan 3	S27

tance due to simplicity, efficiency, relative ease to perform and non-requirement of sequence information (Karp et al., 1997).

RAPD (Williams et al., 1990) has been used for the analysis of genetic variation and identification in *Cassia* species. RAPD markers provide an efficient assay for polymorphism which allows rapid identification and isolation of chromosome-specific DNA fragments. Genetic polymorphism detected with RAPD reveals one allele per locus, which corresponds to the amplification product. RAPD is not expected to identify heterozygous loci. In this context, RAPD is limited. It may be used to identify heterozygous individuals when a single primer generates at least one complementary.

Polymorphic amplification product from each parent (Baird et al., 1992), species and population-specific loci are also identified by this marker. In particular, RAPD is a useful predictive tool to identify areas of maximum diversity and may be used to estimate levels of genetic variability in natural population.

The objective of the present work was to investigate the relationships among 27 indigenous *C. senna* genotypes collected from several regions in Sudan by using RAPD.

MATERIALS AND METHODS

Plant material

27 individuals of *C. senna* from different regions of Sudan were sampled (Table 1).

DNA extraction

Genomic DNA was extracted from fresh leaf tissue of 27 individuals using modified CTAB method (Porebski et al., 1997). The modification was made in intention to improve the quantity and the quality of the DNA. In this method the fine powdered plant materials were immediately transferred into 13 ml Falcon tubes containing 6 ml of pre-warmed lysis solution. Tubes containing the samples were then incubated in a water path at 65ºC with gentle shaking for 30 min and left to cool at room temperature for 5 min. Isoamyl and chloroform mixture (1:24) was added to each tube and the phases were mixed gently for 5 min at room temperature to make a homogenous 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 step of the

Table 2. Polymorphism detected by the use of 6 random primers on 27 Cassia senna individuals.

Name of primer	Sequence of primer (5'- 3')	Total number of bands	Number of Polymorphic bands	% of polymorphic bands
OPA-03	AGTCAGCCAC	7	6	83.3
OPA-9	GGGTAACGCC	5	5	100
OPA-20	GTTGCGATCC	5	5	100
UBC-101	GCGGCTGGAG	6	5	80
UBC-104	GGGCAATGAT	4	3	66.7
UBC-155	CTGGCGGCTG	4	3	66.7
Total		31	27	496.7
Average		5.2	4.3	82.8

chloroform : isoamyl alcohol extraction was repeated twice. The nucleic acids in the aqueous phase were precipitated by adding equal volume of deep cooled isopropanol. The contents were mixed gently and collected by centrifugation at 4000 rpm for 10 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 (10 mM Tris, 1 mM EDTA, pH 8) and stored at -20ºC for further use. The extracted DNA samples were observed under UV illumination after staining with ethidium bromide and agarose gel electrophoresis. The purity and the concentrations of the DNA were then spectrophotometrically assessed following Sambrook et al. (1989) method.

RAPD analysis and primer selection

Several primers were used for PCR amplification. Six primers that produced strongly amplified polymorphic bands with these test templates were selected for RAPD-PCR analysis (Table 2). The PCR reaction mixtures were prepared in 25 µl volumes containing 2.5 µl of 10X Taq buffer, 1.5 µl MgCl2 (50 mM) , 2.5 µl dNTPs (2mM/µl), 2 µl random primer (10 pmol/ µl), 0.5 µl Taq DNA polymerase (5 U/µl) and 1 µl of the extracted DNA (10 ng). The mixture was made up to 25 µl by addition of sterilized distilled water. RAPD/PCR reactions were optimized initiated using an Applied Biometra thermalcycler programmed to repeat the thermal profile. Setting of the PCR program based on three steps. Step one, was an initial denaturation step at 94℃ for 5 mins. Step two, was run for 40 cycles, each starting with denaturation at 94℃ for 1 min, followed by annealing 36ºC for 1 min and ended by extension at 72ºC for 1 min. Step three, was a final extension cycle performed at 72ºC for 7 minutes. The PCR machine was adjusted to hold the product at 4ºC.

The PCR product was mixed with 3 µl of loading dye (0.25% bromophenol blue, 0.25% Xylene Cyanol and 40% Sucrose, w/v) and spun briefly in a micro centrifuge before loading. The PCR products and 1 kp DNA ladder were electrophoresed using 2% agarose gel at 100 volts followed by staining with ethidium bromide then separated fragments and were visualized with an ultraviolet (UV) transilluminator.

Data analysis

For each primer, the number of polymorphic and monomorphic bands was determined. Bands clearly visible in at least one genotype were scored (1) for present, and (0) for absent and entered into a 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 and Lei, 1979). Coefficient of similarity trees were produced by clustering the similarity data with the unweihted pair group method using statistical software package STATISTCA- SPSS (Stat soft Inc.).

RESULTS AND DISCUSSION

For the isolation of good quality DNA, a CTAB-based procedure was optimized in the present study that yield high-quality DNA free of phenols, which may inhibit the activity of Taq polymerase.

In this study, arbitrary 10 mers primers were obtained from the university of British Columbia Biotechnology laboratory and Operon Technologies (Table 2).

Several primers were tested with the 27 genotypes (*Cassia senna*). The result indicate that 6 primers (60%) show at least one consistent polymorphic band. The six informative primers were selected and used to evaluate the degree of polymorphism and genetic rela-tionships within and between all genotypes under study. Total of 31 amplified fragments were distinguished across the selected primers and the statistical analysis showed 27 polymorphic bands among the 27 genotypes with an average of 4.3 polymorphic bands per primer. The maximum numbers of fragment bands were produced by the primer OPA-3 (7 bands) with 83.3% polymorphism while the minimum numbers of fragments were produced by the primer UBC-104 and 155 (4 bands) with 66.7% polymorphism. Pattern of RAPD fragments produced by the 10-mer primer OPA-3, OPA-9, OPA-20, UBC-101, UBC-104 and UBC-155 as shown in Table 2 (See also Figure 1).

The dendrogram showed two main clades (Figure 2). The first clade contained three groups, the first group contained S6 and S7 as sisters also S3 and S4 showed genetic closeness where in the other (S1, S2) and S8 was genetically distant from S3, S4 and S6, S7. The second clade contained two groups where the first contained (S15, S16) which shows closeness and (S18, S21) as sisters also (S19, S20). The second group contained

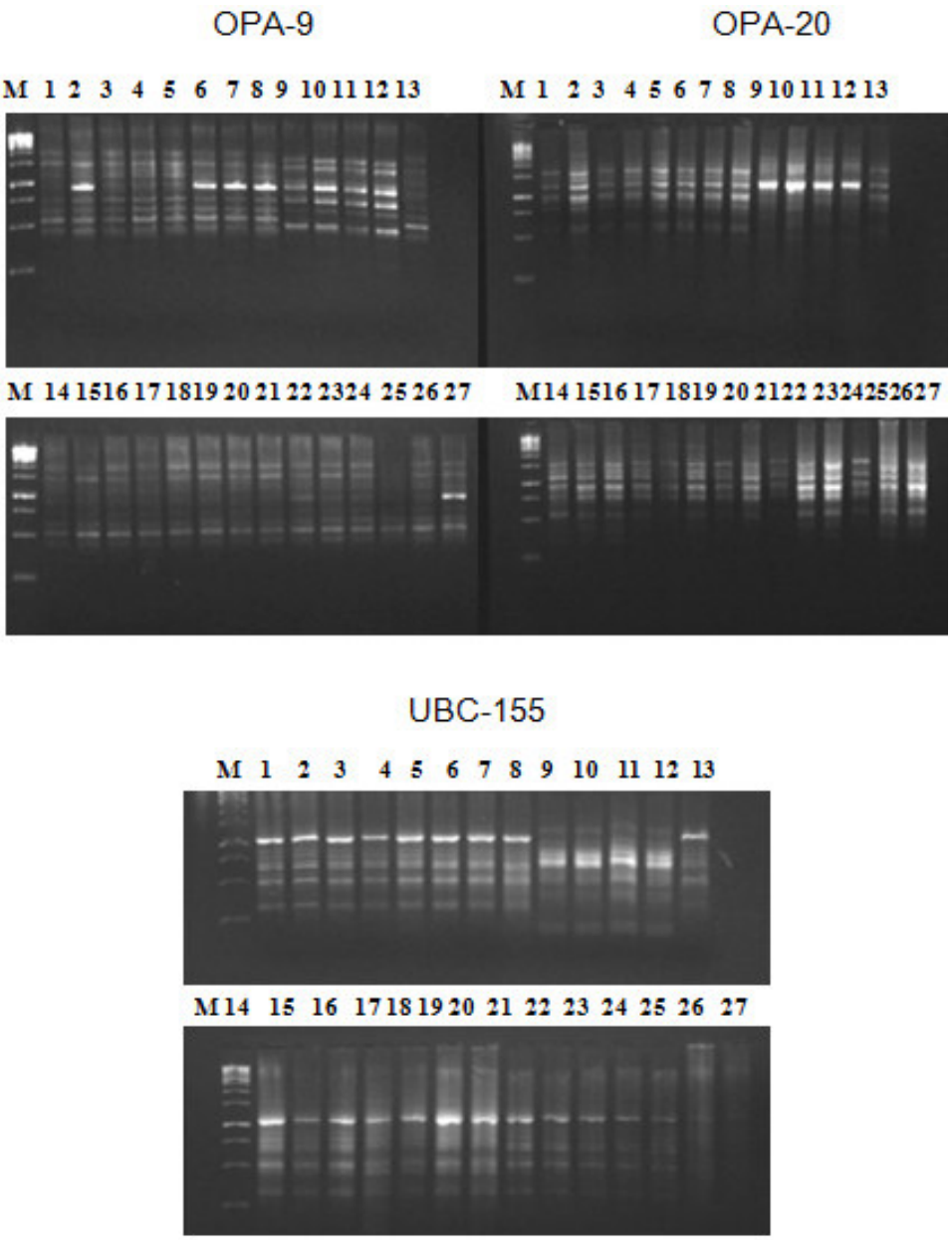

Figure 1. RAPD amplification patterns with primers OPA-9, OPA-20 and UBC-155, (From left to right: lanes 1, 27 and M- ladder 1Kb).

(S23, S24) and (S26, S27), S25 came in a different group. S9, S10, S11 and S12 all from same population (Algizara) came out of group.

Kumar et al. (2007) studied the genetic variation between *Senna surattensis* Burm, f. and *Senna sulfurea* DC. Ex Collad, where they found a species-specific marker distinguishing both species. They concluded that RAPD technique was very useful. This study provides us with good knowledge about genetic variability of *C. senna* which may allow more efficient and effective use of resources in plant improvement programs. In this study we utilized RAPD markers for better assessment of relationship of the accessions of *Cassia* collected from different locations. 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 (Karp et al., 1997), introduces problem in their use for genotype identification compared with other marker applications. This study clearly showed that it was possible to analyze the RAPD and for correlating their similarity and distance between *C. senna* genotypes. From the results of the RAPD profiling, it was observed that *C. senna* genotypes produced good ampli-

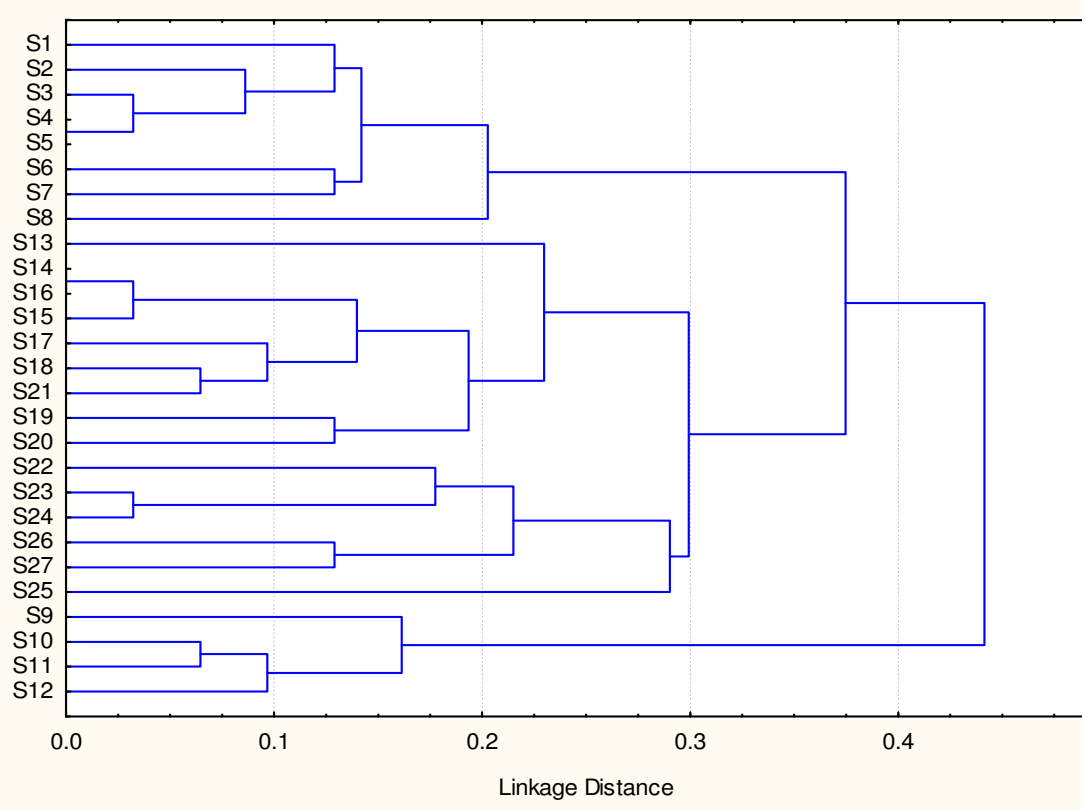

Figure 2. Dendrogram constructed for 27 *C. senna* genotypes based on genetic distances using 6 RAPD primers.

fication but few showed less number of amplified bands on some primers.

The studied individuals showed variation in morphological and characteristic of *Cassia*. However; morphological variation does not always reflect real genetic variation which might be attributed to the control of interaction of the genotype with environment by unknown genetic traits (Smith and Smith, 1992).

Molecular markers can be used to study the genetic diversity and genetic relationships among *C. senna* at the DNA level. The present investigation of DNA profiling in *Senna* species clearly demonstrates that it is possible to analyze the RAPD patterns for correlating their similarity and distance between species and accessions, by which one can predict the origin of the species to great extent.

In conclusion, RAPD analysis revealed high levels of genetic variability, even with the use of limited set of primers. This high level of polymorphism among individuals suggests that RAPD techniques can be useful for *C. senna* for the maintenance of germplasm banks and the efficient selection of parents for breeding.

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