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Genetic diversity analysis of Sorghum (Sorghum bicolor L. Moench) accessions using ISSR markers

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Abstract: The gene pool of the genus Sorghum is characterized by abundant diversity. The immense of morphological diversity of the cultivated races of sorghum had resulted from variable climatic and geographical exposure in which its wild ancestors evolved, coupled with selection pressure imposed by the environment and the man during and after domestication. Understanding and management of the natural variation within the domesticated cultivars and their wild relatives of a plant species is very important in the establishment of an efficient breeding program aimed at crop improvement. The genetic relationship among ten sorghum species, namely, Dorado9 Fitritay, Shahla, Gazan Abead, May sodany,H-306, Giza113, Ezraa-7, Omani Mahali, Boifuel was analyzed by inter-simple sequence repeat (ISSR) method. The results showed that the diversity of sorghum was high at DNA level. Twenty-one primers selected from ISSR primers could amplify 95 clear and reproducible bands, of which 93 bands were polymorphic, accounting for 96.51%. The average of Polymorphic information content (PIC) was 0.4132.The UPGMA clustering associated the varieties into two major clusters, separating African and Syrian varieties, the other major clusters included Asian varieties. Grouping of varieties by UPGMA cluster analysis correlated with the geographical origin, pedigree information, yielding type and agronomic traits indicated that ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among sorghum varieties.
Keyword: Sorghum, varieties, ISSR, PIC, UPGMA.

Introduction

Sorghum (Sorghum bicolor L. Moench) 2n =2x =20] is the fifth most important cereal grain crop after wheat, rice, maize and barley, originated from West Africa and a staple food for millions of poor people in semi-arid tropics of Africa and Asia[1]. It has gained it's importance as a fodder (green/dry) and feed crop in the last decade. Besides being an important food, feed and forage crop; it provides raw materials for the production of starch, fiber, dextrose syrup, biofuels, alcohol and other products. The stems are desired for food grade syrup, fresh chewing and alcohol production in Brazil[2], India and Africa[3].Sorghum is one of the crop species that can survive the harsh climatic conditions of the arid environments[4]. Sorghum belongs to C4plant characteristic for tolerance of abiotic stresses more than many other crops[5].

Sorghum has many types of cultivated varieties, such as grain genotypes, fodder, fiber and sugar genotypes and wild races and possess a significant amount of genetic diversity for traits of agronomic importance[6].

Sorghum yield is limited by inadequate and/or erratic rainfall, poor soil fertility, pest and disease invasions and high temperatures[7]. It is important to increase the sorghum yield to meet the growing demand due to expanding population. In plant breeding programs, assessment of genetic relationship is useful for determining the uniqueness and distinctness of a phenotype, genetic constitution of genotypes and selection of parents for hybridization[8].

DNA markers have been used to evaluate genetic diversity in different crop species[9]. Molecular markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant[10.]

The polymerase chain reaction (PCR) is widely used in genomic DNA analysis. One of its main applications has been in the development of DNA markers for map construction, which are useful in breeding, taxonomy, evolution and gene cloning.

Several PCR marker systems are available varying in complexity, reliability and information generating capacity. These include random amplified polymorphic DNA (RAPD), simple sequence repeat polymorphism (SSR), amplified fragment length polymorphism (AFLP) and a few others[11, 12]. Each system has its own advantages and disadvantages.

Inter Simple Sequence Repeat (ISSR) markers, developed by[13] based on the amplification of a single primer containing a microsatellite 'core' sequence anchored at the 5' or 3' end by a set of 2-4 purine or pyrimidine residues, offers a high degree of reproducibility with the detection of the rich level of polymorphism in a relatively simple procedure. Hence, it has been widely used in assessment of genetic diversity and cultivar identification[14]. ISSR has been successfully used to estimate the extent of genetic diversity at inter and intra specific level in a wide range of crop species which include rice, wheat, fingermillet, Vigna, sweet potato and Plantago[15].

Molecular characterization of twelve varieties of sorghum was carried out with 11 RAPD and three ISSR primers. Out of the different anchored primer combinations, ISSR primers with TC and GA motifs produced clear and maximum scorable bands, thus revealing a better coverage of the genome. Genetic diversity parameters [viz., average and effective number of alleles, polymorphic information content (PIC)] were calculated for both RAPD and ISSR markers

Genetic variations detected among the geographically different populations of sorghum could be of much use for the introgression of new characters from wild counterparts to the cultivars, isolation of stable segregating markers and selection of improved varieties and conservation of germplasm resources[16.] Inter Simple Sequence Repeat (ISSR) markers were used to assess the genetic diversity of 10 sorghum varieties, nine of Indians and one of Syrian origins. Out of 20 ISSR primers screened, 9 primers were selected for their polymorphic and repeatable fragments. 110 fragments were polymorphic out of a total 130 fragments a, while the percentage of polymorphic bands value ranged from 63.6.% of (AC)8 T to 100% of (AG)8 A with a mean of 84.61%. As a result poly (AG)-anchored primers were more polymorphic and reproducible than other di-nucleotides and tri-nucleotides motifs. The UPGMA clustering associated the varieties into two major clusters, separating Indian varieties from the Syrian variety. Among the Indian varieties two sub-clusters were related to the yielding type and maturity date. Grouping of varieties by UPGMA cluster analysis correlated with the geographical origin, pedigree information, yielding type and agronomic traits indicated that ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among sorghum varieties[17.]

50 sorghum accessions with important agronomic traits, representing 11regions in Sudan and Republic of South Sudan were assayed for polymorphism using Inter-simple sequence repeat (ISSR). Seven primers out of 41 tested (807, 808, 810, 814, 848, 872 and 879) showed high polymorphism among the accessions. The results indicated 75 polymorphic bands out of 78 bands with percentage of polymorphic bands of 97%. UPGMA result showed ISSR distance matrix ranged between (0.04-0.47) reflecting high genetic diversity. The ISSR UPGMA dendrogram showed high molecular variance within regions. Based on the results of this study ISSR technique showed differences among closed related accessions of Sorghum[18.].

Material and Methods

1. Plant Materials

Ten varieties of sorghum obtained from ACSAD. (Table. 1) were selected to evaluate the genetic diversity and relationship among their seeds. Table (1)

Table (1). The Names and regions of sorghum accessions used in the study.

Variety No.	Name	Regions
1	Dorado9	Egypt
2	Fitritay	Egypt
3	Shahla	Saudi Arabia
4	Gazan Abead	Saudi Arabia
5	May sodany	Sudan
6	H-306	Egypt
7	Giza113	Egypt
8	Ezraa-7	Syria
9	Omani Mahali	Sultanate of Oman
10	Boifuel	Mauritania

(Dorado9, Fitritay, Shahla, Gazan Abead, May sodany, H-306, Giza113, Ezraa-7, Omani Mahali, Boifuel)

2. Seeds Sterilization

Sorghum seeds were soaked in 70% ethanol for 30 seconds, they were transferred to four vessels which contain sterile distillated water for 5 minutes, then they were put for 5 minutes5% Hyposodiumchloride, then they were soaked three times in distilled water, each for 5 minutes, then planted inpots. Fresh leaves were taken after2-3 weeks to extract the DNA.

3. DNA extraction

DNA was extracted from fresh leaf tissues of sorghum accessions using modified CTAB method[19]. The modification was made in intention to improve the DNA quantity and the quality. In this method the fine powdered plant materials were immediately transferred into 15 ml Falcon tubes containing 5 ml of pre-warmed lysis solution. Tubes containing the samples were then incubated in a water bath at 60ºC with gentle shaking for 30 min and left to cool at room temperature for 10 min. Chloroform: Isoamylalcohol mixture (24:1) was added to each tube and the phases were mixed gently for 10 min at room temperature to make a homogenous mixture. The cell debris was removed by centrifugation at 4000 rpm for 15 min and the resulted clear aqueous phases (containing DNA) were transferred to new sterile tubes. The step of the chloroform: isoamylalcohol 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. The remained ethanol was removed by leaving the pellet to dry at room temperature. The pellet was dissolved in TE buffer (10 mMTris, 1 mM EDTA, pH 8) and stored at -20ºC for further use.

4. DNA Quality and Quantity

The extracted DNA samples were observed under UV illumination after 104 staining with 105 Ethidium Bromide and agarose gel electrophoresis. The purity and the concentrations of the 106 DNA were then Spectrophotometrically assessed following [20]method.

5. PCR of the ISSR Technique

Forty one ISSR primers Table(2)were tested for amplification using the polymerase chain reaction (PCR) in a final volume of 25 µL containing 1.0 µL (25ng) DNA diluted, 0.5 µL (2.5U) Taq polymerase, 2.5 µL 10X buffer, 2.5 µL (2mM/µl) dNTPs, 1.5 µL (50 mM) MgCl2, 2.0 µL (10 pmol/ µl) ISSR primer and 15 µL

ddH2O. The amplifications were performed in a thermal cycler following the program: 94°C for 5 min, 40 cycles (1 min at 94°C, 1 min at 43°C and 1 min at 72°C) and final elongation of 7 min at 72°C.

6. DNA Documentation

In order to load the samples on the 2%agarose gel stained with (2µl (10mg/ml)) Ethidium Bromide, 4 µl of PCR product of each sample were mixed with 2 µl of loading dye and loaded.1.4 µl of 1 Kbp DNA ladder was used. Electrophoresis was done at 80 Volts. The separated fragments were visualized with an ultraviolet (UV) Trans illuminator.

7. ISSR Data analysis

The numbers of polymorphic and monomorphic bands were determined for each primer. Genotypes were scored (1) for present band, and (0) for absent band and then entered into a data matrix[21]. Percentage of polymorphism was calculated as the following equation: (polymorphic bands/total number of bands x 100). The tree diagram was produced by clustering the similarity data with the UPGMA method using POPGENE software V1.31[22].

A PIC (polymorphic information content) value was calculated according to the equation:

PIC =[Σ2(PI(1- PI)^2]

Whereas: PI bands frequency resulting from using the primers in all samples[23].

Table(2) codes, sequences and annealing temperature of used primers:

Annealing temperature	nucleotide sequences	Primer code
52	(AG)8T	ISSR-1
52	(CA)8A	ISSR-3
52	(AC)8T	ISSR-5
52	(GA)8CG	ISSR-6
52	(TC)8GA	ISSR-7
56	(AC)8AG	ISSR-8
52	(AC)8GG	ISSR-9
56	(CA)11	ISSR-13
56	CCAG(GT)7	ISSR-14
56	(GT)4(GA)5	ISSR-15
56	(AC)7(TA)2A	ISSR-16
52	(AG)8C	ISSR-23
51	(AGG)6	ISSR-25
51	(CT)8G	ISSR-26
56	(CA)6ACAG	ISSR-28
56	(TC)8C	ISSR-30
52	(AG)8T	ISSR-32
52	(GA)8T	ISSR-33
52	(TC)8C	ISSR-36
52	(TG)8G	ISSR-37
52	(AC)8TT	ISSR-40
52	(AC)8GG	ISSR-41
52	(TG)8AA	ISSR-43

Results and Discussion

The concentrations of the extracted DNA were between 0.73-1.53 µg/µl, and the purity of the samples was between 1.931-2.080. Electrophoresis was applied on 0.8% agarose gel to know the quantity of DNA

ISSR technique was applied by using 23 primers for amplification of the 10 accessions of sorghum, 21 primers proved their ability in giving polymorphic ISSR products from the studied samples, while 2 primers didn't give any PCR products. ISSR primers were able to distinguish between studied pear varieties, which corresponded with [24].

The studied varieties were tested, (table 3)(Figure 1)showed that the primers gave PCR products of about 95 bands, most of them were polymorphic, and the percentage of polymorphism was %96.51 The bands of each primer rated between 2band as a minimal number for primer (8), and 6 bands for primers (30, 36, 37) as a maximal number of bands, with an average of 7.2 bands/ primer. Polymorphism percentage rated between 66.66%,80% for primers (15, 1,25), which was the minimal, and 100% for all other primers. Polymorphic information content values (PIC) rated between 0.0525 for the primers (8) and 0.4998 for the primer (25) the average PIC value was 0.4132.

Table (3). Total bands number from used primers, the percentage of polymorphic bands and PIC values

PIC	Polymorphism %	Polymorphic Bands	Total Bands	Primer name
0.4712	80	4	**5**	ISSR-**1**
0.48**00**	100	4	**4**	ISSR-**3**
0.48**00**	100	4	**4**	ISSR-**5**
0.4488	100	5	**5**	ISSR-**6**
0.375**0**	100	4	**4**	ISSR-**7**
0.0525	100	2	**2**	ISSR-**8**
0.42**00**	100	5	**5**	ISSR-**9**
0.4928	100	5	**5**	**1**ISSR-**3**
0.3988	100	4	**4**	ISSR-**14**
0.4902	66.66	2	**3**	ISSR-**15**
0.3988	100	4	**4**	ISSR-**16**
0.42**00**	100	4	**4**	ISSR-**23**
0.4998	80	4	**5**	ISSR-**25**
0.48**00**	100	4	**4**	ISSR-**26**
0.4712	100	5	**5**	ISSR-**28**
0.4712	100	6	**6**	ISSR-**30**
0.4712	100	5	**5**	ISSR**32**
0.3432	100	5	**5**	ISSR-**33**
0.4872	100	6	**6**	ISSR-**36**
0.4992	100	6	**6**	ISSR-**37**
0.4988	100	4	**4**	ISSR-**40**
		93	95	Total
0.4132	96.51	4.42	4.52	Average

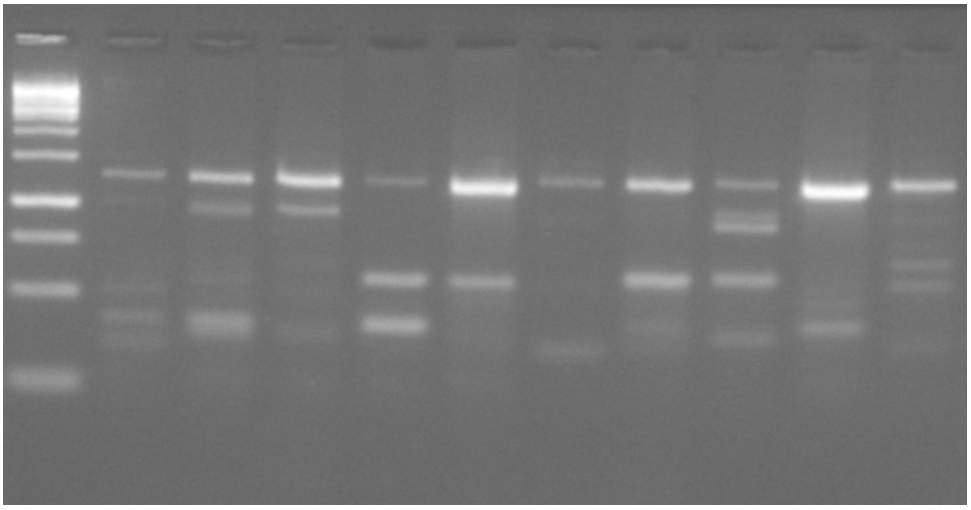

Figure 1. ISSR patterns obtained from the 10 varieties of wheat by using primer ISSR36.

M: 1000 pb molecular weight marker. (1- Dorado9, 2- Fitritay, 3- Shahla, 4- Gazan Abead, 5- May sodany, 6- H-306, 7- Giza113, 8- Ezraa-7, 9- Omani Mahali, 10- Boifuel)

The Percentage of Agreement Values matrix (PAV) for ISSR markers among the ten studied samples ranged from (0.3795) between Fitritay and Dorado9, which were the most closest to each other. The highest PAV value was (0.9163) for example: between Boifuel and Giza113, which were the furthest from each other. Table (4).

Table (4). Percentage of Agreement Values Matrix (PAV)resulted from studying genetic similarity of studied varieties

	Dorado9	Fitritay	Shahla	Gazan abead	May sodany	H-306	Giza 113	Ezraa7	Omany mahali	Boifuel
Dorado9	0.00									
Fitritay	**0.3795**	0.00								
Shahla	0.5465	0.7472	0.00							
Gazan abead	0.6827	0.8162	0.6026	0.00						
May sodany	0.7252	0.7697	0.6419	0.7037	0.00					
H-306	0.6827	0.7697	**0.9163**	0.6221	0.8403	0.00				
Giza 113	0.8903	0.7927	0.5836	0.7252	0.7252	0.6419	0.00			
Ezraa7	0.7927	0.7472	0.6621	0.7252	0.7697	0.6419	0.4763	0.00		
Omany mahali	0.4430	0.5108	0.7037	0.8162	0.6026	0.7697	0.7472	0.7472	0.00	
Boifuel	0.4934	0.6026	0.6419	0.5836	0.6621	0.7927	**0.9163**	**0.9163**	0.8162	0.00

The UPGMA clustering associated the varieties into two major clusters, separating African varieties and the Asian varieties. Among the African varieties two sub-clusters were include Egypt verities (Dorado9, Fitratay) which were the closest to each other (19.43), and the Syrian variety (Eizra7). But, the Asian verities have been separated into two sub-clusters (6.32) the first one contained only "Omani mahali", the other cluster which contained (Gazan abead and Shahla) both are from Saudi (14.59).

Grouping of varieties by UPGMA cluster analysis correlated with the geographical origin, and agronomic traits indicated that ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among sorghum varieties(Figure 2).

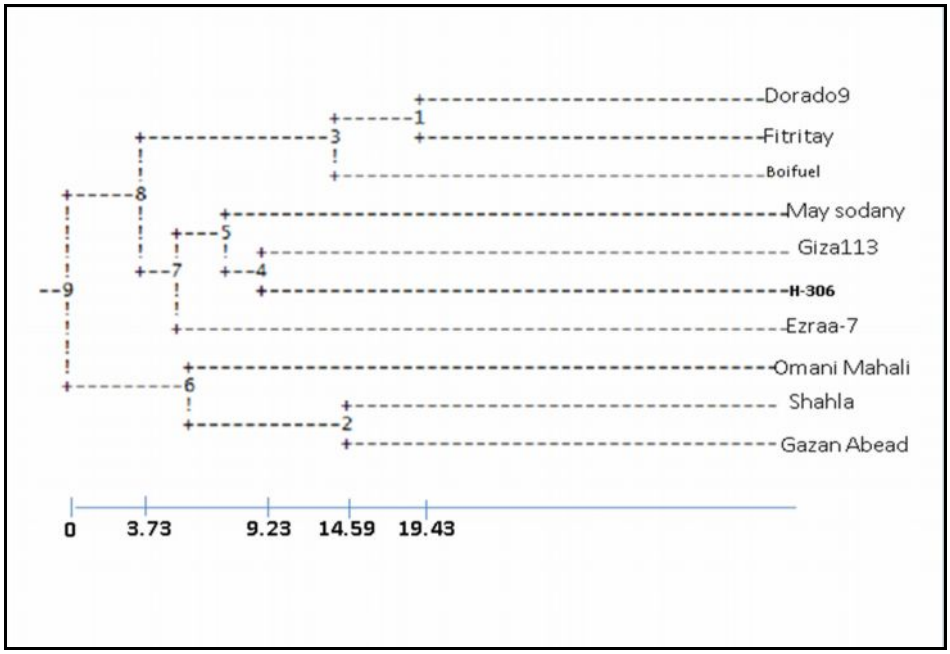

Figure. 2: UPGMA based cluster tree of 10 sorghum varieties with23 ISSR markers.

Conclusions:

1. The ISSR technique proved its efficiency in differentiating the examined varieties which were grouped according to their geographical distribution.
2. This facilitates and enhances their use as promising parents in sorghum breeding and improvement.

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