Genetic diversity among *Sorghum bicolor* genotypes using simple sequence repeats (SSRs) markers

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ABSTRACT

The technique Simple Sequence Repeats (SSRs) was applied to quantify the genetic diversity among nine Sorghum bicolor genotypes of different origins cultivated in Egypt using nine Sorghum bicolor*-derived simple sequence repeat (SSR) markers. The total number of alleles detected by SSR was 70 with an average of 7.3 alleles per primer. The results indicate that 58% of the SSR markers were polymorphic. Cultivar-specific SSR markers characterizing different genotypes were used to generate unique fingerprinting for each genotype. Nine unique positive and negative cultivar specific markers were detected. The unique specific markers characterized 4 out of 9 cultivars. The cluster analysis of SSR data showed a wide genetic background in the examined cultivars. Based on the data reported here, SSR markers appear to be particularly useful for the estimation of genetic similarity among the relatively poorly characterized genotypes of sorghum of different origins.*

Key words: *Genetic diversity, fingerprint, Sorghum, SSR.*

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], a tropical plant belonging to the family Poaceae, is one of the most important crops in Africa, Asia and Latin America (Anglani, 1998) and it is the fifth in acreage among the world cereals (Doggett, 1988). Moreover, Sorghum is the most agronomically important taxon in that it includes the cultivated grain races, it is a diploid, highly self-pollinated, and possesses considerable diversity in morphological and agronomic traits, such as adaptive pest resistance. This wide range of genetic diversity of sorghum suggested the possibility of improving its productivity. The usage of modern techniques such as molecular markers is required to enhance its breeding programs. Many studies have been devoted to assessing the patterns of Sorghum genetic variation based on morphology (Appa-Rao *et al.*, 1996; Djè *et al.*, 1998) or pedigree (Jordan *et al.*, 1998). However, phenotypic variation does not reliably reflect genetic variation because of the role of environmental interaction in determining the phenotype (Smith and Smith, 1989; Smith *et al.*, 1991). In recent years, the number of molecular assays available for application in this area has increased dramatically, with each method differing in principles, applications, type and amount of polymorphism detected, as well as cost and time requirements (Karp *et al.*, 1998). The molecular assays include restriction fragment length polymorphism (RFLP) (Botstein *et al.*, 1980), random

amplified polymorphic DNA polymorphism (RAPD) (Williams *et al.*, 1990), SSR polymorphism (Tautz, 1989), and amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993). Recently, DNA simple sequence repeats (SSRs, known as microsatellites) are becoming the markers of choice for fingerprinting and genetic diversity studies in a wide range of living organisms (Gupta and Varshney, 2000). This technique represents an ideal marker system due to its codominant inheritance, occurs in high frequency, locus specificity, distribution throughout the genomes of all higher plants and animals and multi-allelic characters. It displays a high level of polymorphism, even among closely related accessions, and is amenable to simple and inexpensive polymerase chain reaction (PCR) assays (Brown *et al.* 1996). Radioisotopes are not required in the detection of SSR markers, because sequence polymorphism usually can be detected by separation in agarose gels (Burr, 1994). In addition, SSR fingerprints are generally highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity (Bruford *et al.*, 1998). Therefore, SSRs have been established as useful genetic markers in many plant species (Cregan *et al.*, 1999) and in genetic mapping initiatives for cereals including wheat (Bryan *et al.* 1997; Roder *et al.* 1998), barley (Ramsay *et al.* 2000), rice (Temnykh *et al.*, 2000) and sorghum (Anas and Tomohiko, 2004 and Smith *et al.*, 2000). In Egypt, most of the sorghum cultivars are brought from the International Crops Research Institute for the Semi-Arid Tropics ICRISAT and adapted to the Egyptian environment. In addition, there are some local cultivars developed by the breeding programs at the Field Crop Research Institute, Agricultural Research Center (ARC), Giza, Egypt. The pedigree history of sorghum cultivars grown in Egypt is not known and information of their genetic background and genetic diversity have not been reported yet, at least by using SSR markers. Here, we report on the use of the high-throughput SSR technology for molecular characterization of the Egyptian cultivated sorghums, either locally or of different origins. The main objectives are to estimate genetic diversity and determine the genetic relationships among these cultivars and to compare genetic similarity quantified by molecular markers with regional and race informations.

MATERIALS AND METHODS

Plant materials

Nine cultivars of *Sorghum bicolor* of different origins cultivated in Egypt were evaluated using SSR technique. Cultivars name, their origins and line grouping are listed in Table (1). The seeds of the nine cultivars were kindly provided by the Field Crops Institute, Agricultural Research Center (ARC), Giza, Egypt. This study was carried out in the period from July, 2007 to November, 2007 at the Genetic Engineering Research Center, Faculty of Agriculture, Cairo University.

DNA preparation

Seeds were grown in a growth chamber at 27°C with 12hr d /light. Genomic DNA was isolated from the leaves collected from 10- 15-day old seedlings according to the protocol described by Dellaporta *et al.* (1983).

SSR primers

Nine SSR markers described by Brown *et al.* (1996) and Kong *et al.* (2000) were used for genotyping assays. Primers names, sequences and corresponding annealing temperatures are listed in Table (2).

PCR amplification and electrophoresis

PCR amplification was performed in a volume of 20 µl containing approximately 30 ng of template DNA, 1µM of each forward and reverse primer, 200 µM of each dNTP, 3 mM MgCl2 and 1 U Red Hot Taq polymerase (ABgene Housse, UK) and 10-X Taq polymerase buffer (ABgene Housse, UK). Reactions were conducted in Biometra T1 Thermocycler (Germany) with initial denaturation step for 5 min at 94°C followed by 40 cycles of 94°C for 1 min, 50°C to 55°C (depending on primers, Table 2) for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 7 min. The SSR reaction products were evaluated for polymorphisms on 2% agarose gel. After staining with 1 µg mL^{-1} ethidium bromide for 30 to 60 min, the gels were photographed by Gel Documentation system.

Table (1): The origin and line grouping of the nine sorghum cultivars.

No	Cultivar	Source	Agronomic groups and morphological note
1	NEBdoraoV9	Nebrasca (U.S.A)	Grain, stay green, white seed, short stem and resistant to (*Acremonium strictum*) , (*Fusauium moniliforme*) and *downy mildew.*
2	ICSV237	ICRESAT	Grain, stay green, white seed , dwarf stem
3	Giza 15	Egypt (Local)	Grain, white seed, long stem and(*Acremonium strictum*), (*Fusauium moniliforme*) and *downy mildew.*
4	ICSV112	India	Grain, white seed long stem
5	ICSR93004	ICRESAT	Grain, Stay green, creamy seed and short stem.
6	BTX 631	University of A&M in Texas (U.S.A)	Grain, Stay green, white seed and short stem.
7	ICSR92003	ICRESAT	Grain, Stay green, creamy seed and short stem.
8	ICSB37	ICRESAT	Grain, Stay green, white seed and short stem.
9	NES1007	Near East Sorghum Nursary. Ford foundation organization in 1972	Grain,Stay green, orange seed and short stem.

Band scoring and cluster analysis

The SSR gel images were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v. 4.0.1 (Bio-Rad Laboratories, Hercules, CA USA). The bands were sized and then binary coded by 1 or 0 for their presence or absence in each genotype. The systat ver. 7 computer program was used to calculate the pairwise differences matrix and plot the dendrogram among sorghum cultivars (Yang and Quiros, 1993). Cluster analysis was based on similarity matrices obtained with the unweighed pair-group method (UPGMA) using the arithmetic average to estimate the phenogram.

Table (2): Types and sequences of the SSR loci and annealing temperature for PCR reaction.

Locus	Types of SSR (s)	Sequence of forward primers	Sequence of reveres primers	Ann Temp.
*Xtxp6**	$(CT)_{14}$	ATCGGATCCGTCAGATC	TCTAGGGAGGTTGCCAC	50
*Xtxp7**	$(CT)_{14}$	ACATCTACTACCCTCTCACC	ACATCTACTACCCTCTCACC	50
*Xtxp8**	$(TG)_{21}$	ACATCTACTACCCTCTCACC	ACACATCGAGACCAGTTG	50
*Xtxp10**	$(CT)_{14}$	ATACTATCAAGAGGGGAGC	AGTACTAGCCACACGTCAC	50
*Xtxp12**	$(CT)_{22}$	ATATGGAAGGAAGAAGCCGG	AACACAACATGCACGCATG	55
*Xtxp17**	$(TC)_{14} + (AG)_{12}$	CGGACCAACGACGATTATC	ACTCGTCTCACTGCAATACTG	55
*Xtxp19**	$(AG)_{14} + (AG)_{10}$	ATACTATCAAGAGGGGAGC	AGTACTAGCCACACGTCAC	55
*Xtxp61**	——	GATGCCCATGCCTTGC	CCCACTAAACTAAAGCGACA	55
*Xtxp96**	——	AGATCTGGCGGCAACG	AGTCACCCATCGATCATC	55

RESULTS AND DISCUSSION

SSRs were characterized in different eukaryotic organisms, being the most variable component of the genome with a high rate of molecular evolution. The distribution and sequence of SSR markers may therefore, provide insight into phylogenetic relationships among varieties and species. The microsatellite variation is thought to be due to slippage of the DNA polymerase during replication of unequal crossing over resulting in differences in the copy number of the core nucleotide sequence (Yu *et al*, 1999; Qureshi *et al.*, 2004). In the present study, nine primer pairs (Table 2) flanking dinucleotide simple sequence repeats were employed to investigate the level of polymorphism among the nine sorghum cultivars of different origins and cultivated in Egypt. All primers produced amplicons, even when using modified amplification conditions and all the nine loci were polymorphic with different levels of polymorphism, thus revealing 58% polymorphism (Table 3). In total, 70 alleles were detected in the 9 SSR loci, with an average of 7.3 alleles per locus. The number of amplification products per primer pair varied from 4 to 13, and the size of the amplified fragments ranged from 49-1045 bp which reflected remarkable differences in the number of repeats between the different alleles. The total number of putative alleles at each locus and the observed size ranges of these alleles are given in Table (3). The existence of some minor bands (shadow, heteroduplex and faint bands) may affect the allele scoring process. However, Wang *et al.* (2003) and Rodriguez *et al.* (2001) reported that the minor bands can be useful during gel scoring for genotype

verification, because they are generally consistent. In addition, for some loci the size range of bands obtained in this study are substantially wider than that reported earlier (Kong *et al.*, 2000).

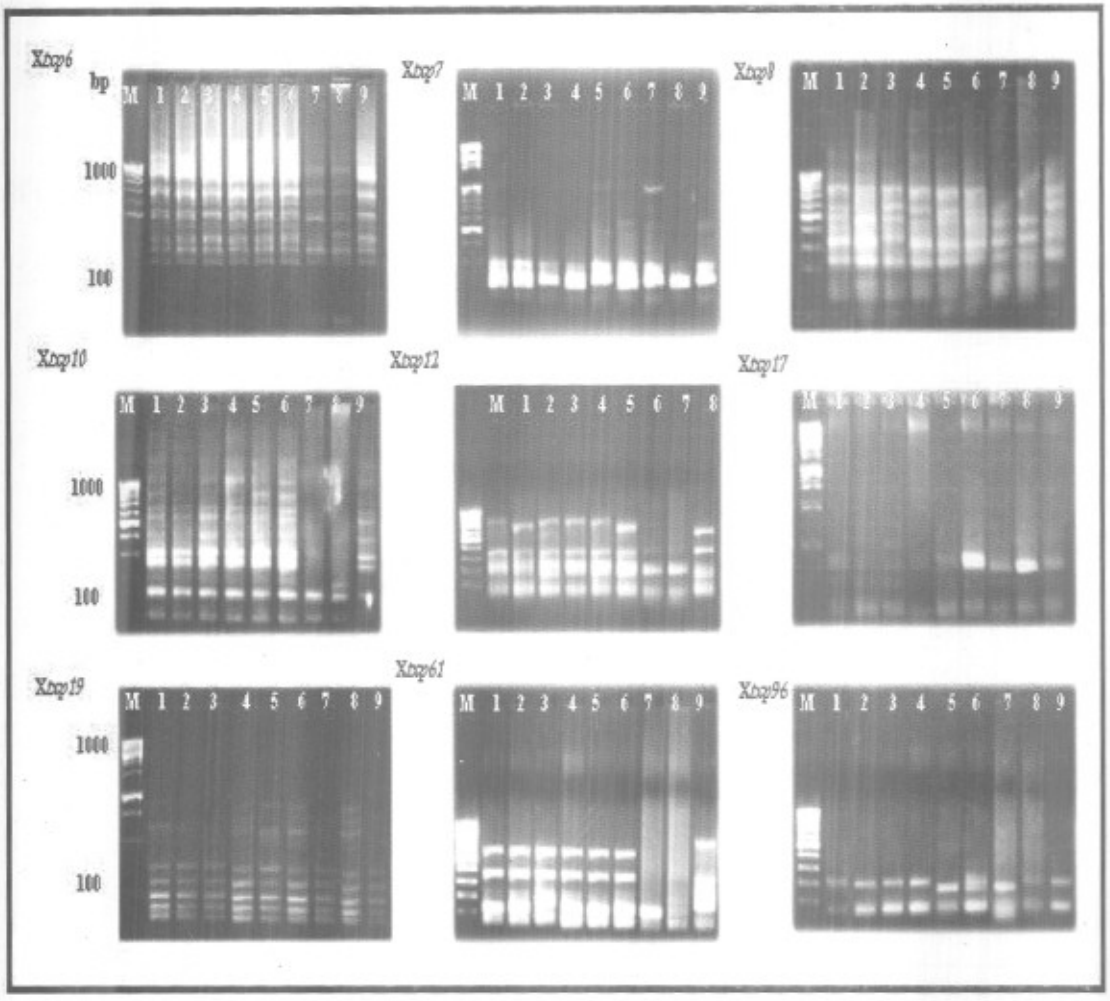

Fig. (1): Profiles of the nine sorghum cultivars as revealed by SSRs. Multiple loci detected by SR primers in the sorghum cultivars. Lanes from: 1 to 9 represent, NESdoradoV9, ICSV273, Giza 15, ICSV 112, ICSR 93004, ATX 631, ICS 93002, ICSB 37 and NES 1007, respectively. M: DNA ladder marker (100-bp).

The variability in the number of alleles per locus (4 to 13) may result from different locus specific mutation rates (Estoup *et al.*, 2002) and reflects strong differences in allelic diversity between SSR loci, which affects estimating genetic diversity and the diversity index (DI). According to Nei (1973), this behavior depends on both the number of alleles per locus and the respective allele frequency (McCouch *et al.*, 1997). Besides

locus specific mutation rates, the number of alleles per locus and gene diversity can be affected (i.e., reduced) by size homoplasy which occurs when different copies of a locus are identical in state, although they are not identical by descent (Estoup *et al.*, 2002). However, microsatellites are typically multiallelic markers (Matsuoka *et al.*, 2002) with heterozygosity values much higher than those of RFLPs (McCouch *et al.*, 1997). Accordingly, different authors have shown that microsatellites with three or more alleles per locus are more common than those with less than three alleles per locus in sorghum (Taramino *et al.*, 1997; Kong *et al.*, 2000) and in maize (Matsuoka *et al.*, 2002).

Table (3): Number of alleles, fragment size range and polymorphism detected by the SSR loci in the nine sorghum cultivars.

Primer	Fragment Size (pb)	No of Alleles	Monomorphic bands	Polymorphic bands	
Xtxp6	**1045 - 0082**	**13**	**6**	**7**	
Xtxp7	**1000 - 0179**	**6**	**3**	**3**	
Xtxp8	**0800 - 0100**	**8**	**5**	**3**	
Xtxp10	**1015 - 0089**	**11**	**2**	**9**	
Xtxp12	**0819 - 0082**	**6**	**4**	**2**	
Xtxp17	**0258 - 0173**	**4**	**2**	**2**	
Xtxp19	**0492 - 0049**	**9**	**5**	**4**	
Xtxp61	**0917 - 0080**	**7**	**1**	**6**	
Xtxp96	**0390 - 0082**	**5**	**1**	**4**	
Total		**69**	**29**	**40**	**58 %**

Genetic diversity and relationships of sorghum cultivars

To examine the genetic relationships among the nine sorghum cultivars under study based on the SSR results, the data scored from the nine primers were compiled and analyzed using the Dice similarity coefficient. The genetic similarity matrices based on the Dice coefficients are shown in Table (4). To estimate the relationships between the Egyptian sorghum cultivar Giza15 and other cultivars of different origins cultivated in Egypt, the data in Table (4) and Fig. (2) showed that the local cultivars are relatively closely related to those of ICSV273 (86.2 %), ICSV112 (84.7 %) and BTx63 (82.5 %) but distantly related to those of ICSR92003 and ICSB37 (54.8 and 55.0%, respectively. Similarities among the 9 sorghum cultivars ranged from 53.3% (ICSV112 and ICSR92003 and ICSR92003 and NES1007) to 91.2% (NEBdoradoV9 and ICSV273). The cultivars of sorghum showed relatively high genetic similarity percentages, i.e., 87.9%, 87,1 and

86.4 were between NEBdoradoV9 and either of ICSR93004, BTX631 or ICSV112, respectively.

Table (4): Genetic similarity (GS) matrices computed according to Dice coefficient from SSRs of the nine sorghum cultivars of different origins.

	NEBDORADOV9	ICSV273	GIZA15	ICSV112	ICSR93004	BTX631	ICSR92003	ICSB37	NES1007
NEBDORADOV9	1.000								
ICSV273	0.912	1.000							
GIZA15	0.820	0.862	1.000						
ICSV112	0.864	0.911	0.847	1.000					
ICSR93004	0.879	0.828	0.800	0.845	1.000				
BTX631	0.871	0.883	0.825	0.900	0.852	1.000			
ICSR92003	0.593	0.596	0.550	0.533	0.596	0.556	1.000		
ICSB37	0.617	0.621	0.548	0.638	0.649	0.656	0.717	1.000	
NES1007	0.719	0.754	0.817	0.714	0.698	0.754	0.533	0.557	1.000

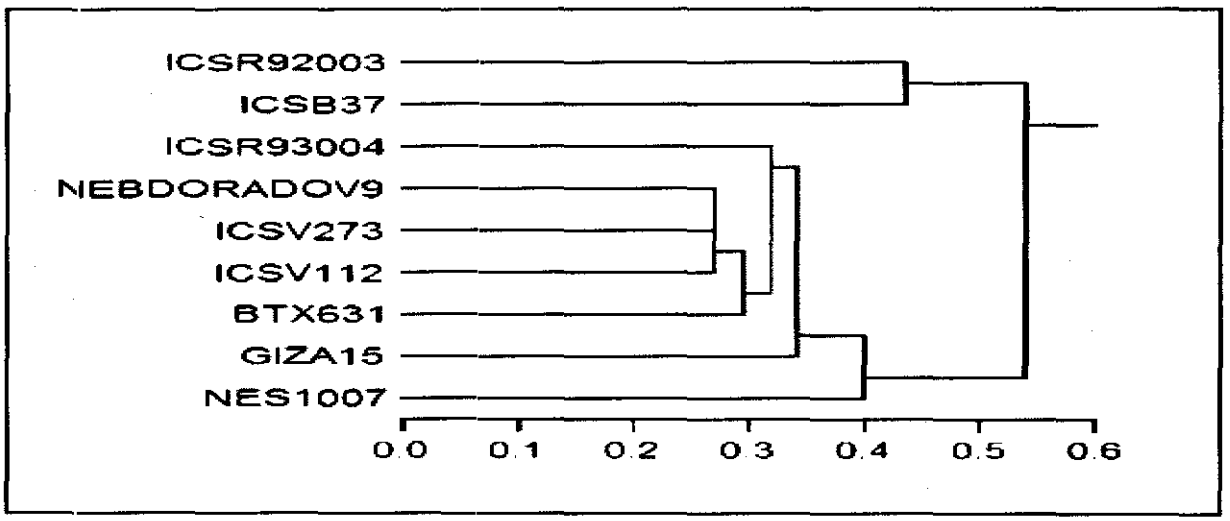

Fig. (2): Dendrogram for the nine sorghum cultivars constructed from SSRs data analysis using Unweighed Pair-group Arithmetic Average similarity matrices computed according to Dice coefficients.

The dendrogram is divided into two main clusters; the first main cluster is divided into two subclusters containing the two ICRIST cultivars ICSR 92003 and ICSB 37. The second main cluster is divided into two subclusters; the first contains only NES1007. The second subcluster is divided into two branches, the first contains only Giza15, the

second is divided to two branches the first comprises ICSR93004, the second has two branches, the first contains BTX 631only, and the second contains three cultivars, (NEBdoradoV9, ICSV273 and ICSV112).

Genotype identification by SSR markers as unique markers

Unique markers are defined as bands that specifically identify varieties from the others by their presence or absence. The bands that are present in one variety but not found in the others are termed positive unique markers (PUM), opposite to the negative unique markers (NUM). Unique DNA markers had been obtained by SSR and were used to characterize the nine sorghum cultivars of different origins. In the present study, SSR gave definite identification of four cultivars of sorghum, i.e. five primers out of the nine revealed nine unique SSR alleles (4 positive and 5 negative) as recorded in Table (5). NES1007 was characterized with the highest number of unique markers (4), two positive with primer Xtxp61 and two negative with primer Xtxp61 and Xtxp96. This was followed by ICSR92003 that was characterized by three unique markers, one positive with primer Xtxp96 and two negative with primers Xtxp10 and Xtxp61.

Table (5): Sorghum cultivars characterized by unique positive and/or negative SSR markers, marker size and total number of markers identifying each cultivar.

Cultivars	Unique positive markers			Unique negative markers			Total
	Size of the marker band (bp)	primer	Total markers / cultivars	Size of the marker band (bp)	primer	Total markers/ cultivars	
NEBdoradoV9	400	Xtxp8	1	-----	-----	-----	1
ICSV112	-----	-----	-----	173	Xtxp17	1	1
ICSR92003	390	Xtxp96	1	380 200	Xtxp61 Xtxp10	2	3
NES1007	917 262	Xtxp61	2	114 140	Xtxp61 Xtxp96	2	4
Total			4			5	9

The two cultivars, ICSV112 and NEBdoradoV9 were characterized with one negative unique marker with primer Xtxp17 and one positive marker with primer Xtxp17, respectively. These unique bands could have a number of potential applications including the determination of cultivar purity, efficient use and management of genetic resources collection and the establishment of property rights.

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الملخص العربي

تحديد الاختلافات الوراثية بين تسعة اصناف من الذرة الرفيعة باستخدام واسمات التتابعات المتكررة البسيطة

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تمت استخدام تكنيك التتابعات المتكررة البسيطة (simple sequence repeats) لدراسة الاختلافات الوراثية بين تسعه اصناف من الذرة الرفيعة من اصول وراثية مختلفة . تم الحصول على ٧٠ واسم SSR و ذلك باستخدام تسعة بوادئ SSR متخصصة للذرة الرفيعة و اوضحت النتائج ان ٥٨% من هذه الواسمات المتحصل عليها كانت متباينة. بدراسة واسمات الSSR المخصصة لكل صنف تم تمييز ٤ اصناف من التسعة تحت الدراسة. بصفة عامة اوضحت هذه الدراسة ان واسمات التتابعات المتكررة يمكن استخدامها بكفاءة فى تحديد الاختلافات الوراثية بين اصناف الذرة الرفيعة المستخدمة و لذلك اهمية كبيرة فى عمليات الانتخاب و برامج التربية.