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Characterization and mapping of simple sequence repeats (SSRs) in *Sorghum bicolor*

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Abstract A total of 13 SSR loci were characterized in *Sorghum bicolor*. Ten of these loci were isolated by screening sorghum genomic AG-enriched libraries with labelled poly(AG)/poly(CT), the other three were derived from database searches. In order to explore the degree of polymorphism detectable in this species by this type of molecular marker, the SSR markers were tested on nine inbred lines of *S. bicolor* of different geographic origin. PCR analysis on acrylamide gels revealed a high degree of polymorphism ($\delta_T = 0.80$). One locus, in particular, allowed the identification of all of the nine inbred lines used in our study. Seven of these SSR markers were mapped, using an existing sorghum RFLP map.

Key words *Sorghum bicolor* · Simple sequence repeats (SSRs) · Polymorphism · Mapping

Introduction

Although *Sorghum bicolor* is a species of main economic importance among the cereal crops cultivated in countries of tropical climates, information regarding its genome organization and mapping is rather limited. However, in recent years, following the introduction of molecular markers in plant genetic research, considerable effort has been made to gain a better understanding of sorghum genetics and evolution, and important data have been gathered.

At least seven RFLP maps of sorghum have been constructed using both DNA probes previously mapped in the maize genome (Hulbert et al. 1990; Binelli et al. 1992; Whitkus et al. 1992) and specific sorghum DNA probes (Chittenden et al. 1994; Pereira et al. 1994; Ragab et al. 1994; Xu et al. 1994). Information on the relationship between and within wild and cultivated sorghum has been obtained by means of either nuclear and chloroplastic RFLPs (Aldrich and Doebley 1992; Cui et al. 1995) or mitochondrial DNA analyses (Deu et al. 1995). The advent of PCR-based molecular marker techniques, such as RAPDs (random amplified polymorphic DNA, Williams et al. 1990) has further facilitated the analysis of the sorghum genome. Pammi et al. (1994) identified conditions that allowed reproducible amplification of RAPDs and tested them on 32 different genotypes of sorghum; Tao et al. (1993) used 29 random oligonucleotide primers on 36 genotypes, but were able to detect only a low level of genetic polymorphism. The discovery and the application of a different polymorphism assay, which is based on the variation of the number of short tandemly repeated DNA sequences (simple sequence repeats or SSRs) detected utilizing pairs of unique primers flanking the repeated region in a polymerase chain reaction, has not only revolutioned mammalian genome analysis (Hearne et al. 1992) but has also facilitated plant breeding and genetics. Recently SSR marker technology has been developed and used for genome mapping and DNA fingerprinting in different plant species, such as rice (Wu and Tanksley 1993; Yang et al. 1994), wheat (Röder et al. 1995), barley (Saghai Maroof et al. 1994), maize (Senior et al. 1993; Taramino and Tingey 1996), soybean (Cregan et al. 1994; Morgante et al. 1994) and tomato (Broun and Tanksley 1996).

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These investigations, based on microsatellites obtained through both database searches and random screening of genomic libraries, have demonstrated that the high level of polymorphism intrinsic to this marker system may improve the genetic analysis of plant species with medium or low genetic variability. Furthermore, the ease and speed of genetic analysis based on SSRs enhance the ability to make a greater number of SSRs available to the scientific community, at least for most of the species of social and economical value, such as sorghum, for which SSRs are not yet available. Due to the initial high cost and time required, the production of a suitable number of SSRs in sorghum, as well as in other species, can only be obtained through the effort of several laboratories. For this reason we undertook a project with the main objective of producing novel SSRs and assessing their value as genetic markers in *S. bicolor*.

Here we report the result of the analysis of 13 SSR loci in *S. bicolor* and their mapping. The polymorphism analysis was performed on a set of nine sorghum accessions, chosen in order to represent the sorghum germ plasm. Segregation analysis was performed on a F_2 population (Pereira et al. 1994) using 7 out of the 13 SSR loci in order to verify the reliability of SSR-derived polymorphism for sorghum genetic mapping.

Materials and methods

Enriched genomic DNA library construction

One-hundred micrograms of sorghum DNA isolated from the inbred line IS9203 was digested to completion with the restriction enzyme *Tsp*509I (New England Biolabs) and then separated on an agarose gel 1:1 Agarose:NuSieve (FMC Bioproduct). A 300–600 bp fraction was recovered on a NA-45 DEAE membrane (Schleicher and Schuell) and enriched for an AG repeat through hybridization and capture with a biotinilated oligo containing a $(CT)_{13}$ repeat (Morgante et al., personal communication). The enriched fraction was cloned into phage Lambda ZapII (Stratagene) according to the manufacturer's instruction.

Hybridization screening for microsatellites

The Lambda Zap library was screened by plaque lifting onto nylon membranes (Hybond N, Amersham), and hybridization to ^{32}P-labelled poly(dA.dG)/(dT.dC) probes as described by Sambrook et al. (1989). The density of the plaques was approximately 500–700 pfu/plate.

Pre-hybridization was performed for 6 h in 1 M NaCl, 50 mM Tris-HCl pH 7.5, 1% SDS, 5% dextran sulphate at 65°C. The overnight hybridization was at the same temperature. Initial washes were performed at room temperature in 2 × SSPE, 0.1% SDS (Sambrook et al. 1989) followed by two stringent washes of 15 min each at 65°C in 2 × SSPE, 1% SDS. Plaques containing SSRs were identified by autoradiography.

An anchored PCR screening strategy on the purified phage stocks, followed by agarose-gel analysis, was used to identify the clones containing SSRs and their position within the insert (Rafalski et al. 1996).

Table 1 *Sorghum* accession lines included in this study

Inbred	Origin	Group
B35	Ethiopia	durra
BT×378	(USA) derived	kafir
CSM63	Mali	conspicuum
Da-She-Yan	China	kaoliang/narvosum
Hegari (PI34911)	Sudan	caudatum/kafir
RT×2817	Ethiopia	zera zera
RT×7000	(USA) derived	milo (subglabrescens)
SC279-14E	West Africa	conspicuum
SC326-6	Ethiopia	nigaricans

Double-stranded pBluescript DNAs obtained after in vivo excision and plasmid DNA preparation (Wizard Minipreps, Promega Co., Madison, Wis.), were manually sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, Ohio). The sequence products, labeled with ^{35}SdATP, were run on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

Plant material

Nine sorghum inbred lines of different geographical origin and race (Table 1), were used for polymorphism analysis. DNA was extracted from leaf tissues according to the procedure of Dellaporta et al. (1985).

Segregation analysis was performed on DNA of 68 F_2 individuals from the cross CK60 × PI 229828 (Pereira et al. 1994).

Polymerase chain reaction

Primer pairs for SSR amplification were designed using the PRIMER program provided by E. Lander (Cambridge, Mass., USA)

The PCR reactions were performed and analyzed as described by Taramino and Tingey (1996) except for the annealing temperature of 54°C.

Data analysis

The degree of differentiation, δ_T (Gregorius 1987), of the population represented by the sorghum inbred lines described in Table 1, was computed as:

$$\delta_T = \left[1 - \sum(p_i)^2\right] (N/N - 1),$$

were N is the population size and p_i is the frequency of the ith allele in the population studied for each SSR locus.

For genetic mapping, analysis of the segregation data was accomplished as described by Pereira et al. (1994).

Results

Identification of sorghum SSR loci

A sorghum genomic DNA library with small insert size (200–600 bp) was constructed after enrichment for AG repeats through hybridization and magnetic capture of size-fractionated DNA (Morgante et al., personal communication).

Table 2 Primer sequences, flanked repeats and annealing temperature of the 13 SSR markers loci used

SSR name	Flanking sequence	Annealing tmperature	Repeats
SbAGA01	CGAACCATGATAAATGACTG ATCCGTTTCACAAAAAAGT	54°C	$(AG)_{33}$
SbAGE01	GACCGATCTAATGATGCAG ACGGTAGAGAAGACCCATC	54°C	$(AG)_{30}$
SbAGB02	CTCTGATATGTCGTTGTGCT ATAGAGAGGATAGCTTATAGCTCA	54°C	$(AG)_{35}$
SbAGD02	AGCACTGCTTGACACTCC CTTTGAAACCCTGAACTCAT	54°C	$(AG)_{32}$
SbAGG02	ATCCATGCATATATTCCGAC TTCGTCACCCACAACATAC	54°C	$(AG)_{41}$
SbAGB03	GTGTGTGTAGCTTCTTGGG ACGTAGGAGTAGTTTCTAGGATT	54°C	$(AG)_{41}$
SbAGE03	AGCTCTCAGCCTTTCACAAT GGAAGAAAGGAATGACTTGA	54°C	$(AG)_{34}GA(CA)_4$
SbAGF06	GTTAAACGACCAATCACCC TAGAGGTGTCACTGATGAGC	54°C	$(AG)_{35}$
SbAGF08	ATGGTCGTCTGTCCAGGT CAGTTGCTAATCTTTGACCG	54°C	$(AG)_{34}$
SbAGH04	GGCACTCATGGAGTCACA TTTATCCAAATCAAACCGG	54°C	$(AG)_{39}$
SvPEPCAA	GCAGCTCAGGGACAAATAC CTGCTTCAGGTAAGGATCG	54°C	$(AT)_{10}$
SvHPRGPG	ACTCCGACGCACCCTAAG CTCCATTCTTGTAGCACGTA	54°C	$(CGC)_8$
SbKAFGK1	AGCATCTTACAACAACCAAT CTAGTGCACTGAGTGATGAC	54°C	$(ACA)_9$

About 20% of the clones proved positive after screening with radiolabelled poly $(AG)_n$. With one round of plaque hybridization, 48 clones were recovered and further characterized by Anchored PCR (Rafalski et al. 1995) to confirm the presence of the expected repeat, to determine the SSR position inside the cloned fragment and to choose the better clones to be sequenced. About 90% of the isolated clones contained the expected SSR sequence and 70% of these were neither too far nor too close to the cloning site. The sequences obtained from 20 clones revealed the presence of quite long AG repeats, ranging from 22 to 41, with an average of 34 dinucleotide repeats.

After sequencing, primers were designed for 15 SSR loci. Primers were designed to produce PCR amplification products at the annealing temperature of 54°C and all were verified through PCR amplification of genomic DNA from the sorghum line IS9203. PCR products were visualized by separation on a 3.5% Metaphor agarose gel. Ten of the tested primer pairs gave a good amplification pattern and were selected for further study.

Searches for the presence of di- and tri-nucleotide repeated motifs in sorghum DNA sequences within GenBank and EMBL databases, allowed the identification of three additional SSR-containing loci. An $(AT)_{10}$ repeat was found in the 9th intron of the Phosphoenolpyruvate carboxylase gene (SvPEPCAA, accession number S39756), a $(CGC)_8$ in the coding sequence of the hydroxyproline-rich glycoprotein (SvHPRGPG, X56010), and a $(ACA)_9$ repeat in the coding sequence of the Kafirin locus (SbKAFGK1, X16104). Three pairs of primers were designed using the same criteria previously reported for the ones isolated through library screening.

Table 2 lists the sequence of the primers defining the 13 SSRs, along with the annealing temperature and the nature of the repeat.

Polymorphism analysis

The 13 primers produced were used to amplify the DNA from nine sorghum inbred lines of different geographic origin and race (Table 1).

The allele composition of each line was determined by analyzing each amplified product on 6% denaturing acrylamide gels after end-labelling of one of the two primers with ^{33}P. Figure 1 shows an example of the length polymorphism depicted with one primer set.

Results from the polymorphism analysis are summarized in Table 3 in which the expected PCR product size is referred to the original sequence of sorghum line IS9203 (SbAG clones) or to the sequences retrieved from the database. The range of allele size is also reported, along with the number of alleles detected at each locus; 70% of the primers detected at least five alleles. A minimum of three alleles was detected using the primers SbAGE03 and SvHPRGPG, while one primer (SbAGE01) allowed us to detect nine different alleles.

As a measure of variability, in order to estimate the information content of our set of SSR loci, we chose δ_T

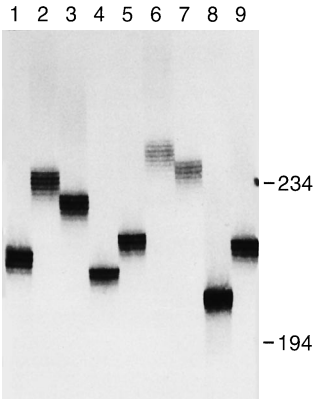

Fig. 1 Autoradiogram of a denaturing polyacrylamide gel with PCR-amplified alleles in nine sorghum inbred lines using the primer pair SbAGE01. *1* = B35, *2* = BTX378, *3* = CSM63, *4* = Da-She-Yan, *5* = Hegari, *6* = RTX2817, *7* = RTX7000, *8* = SC279-14E, *9* = SC326-6

Table 3 Sorghum SSR primer pairs used for polymorphism evaluation. The expected PCR product length and the observed range are reported in columns 2 and 3. Columns 4 and 5 show the number of alleles detected and the δ_T values calculated for each locus after analysis on a 6% polyacrylamide gel of the nine sorghum lines described in Table 1

Primers	Expected PCR product size	Observed PCR product range	No. alleles	δ_T
SbAGA01	116	88–106	6	0.89
SbAGE01	232	208–240	9	1.00
SbAGB02	149	101–121	3	0.75
SbAGD02	134	100–148	6	0.89
SbAGG02	190	170–190	6	0.84
SbAGB03	126	94–158	5	0.72
SbAGE03	149	81–151	3	0.46
SbAGF06	150	110–180	5	0.69
SbAGF08	164	134–176	7	0.92
SbAGH04	152	110–170	8	0.98
SvPEPCAA	206	210–250	7	0.94
SvHPRGPG	246	246–255	3	0.51
SbKAFGK1	142	142–166	4	0.80

(Gregorius 1987), which depends on the number of alleles detected and the distribution of their frequency taking into account the finite dimension of the population under study. The level of differentiation, as expressed by δ_T, for each SSR locus considered is shown in Table 3. Its values range from a minimum of 0.46 for the marker SbGE03 to a maximum of 1 for SbAGE01, which allowed us to discriminate each of the nine sorghum genotypes. The arithmetic mean of δ_T, 0.80, can be a good index of the information content of this kind of molecular marker and proves the utility of identifying and using SSR-derived polymorphism for the genetic analysis of *S. bicolor*.

Linkage analysis

Linkage analysis was performed to determine the distribution of SSR-containing loci in the sorghum genome. After a preliminary screening for polymorphism, seven primer pairs were used to amplify DNA from 68 F_2 individuals derived from the original cross between CK60 and PI22898 (Pereira et al. 1994). The amplification products were run on a 4% Metaphor gel and the segregation data analyzed using MAPMAKER (Lander et al. 1987). Figure 2 shows the position of the seven SSR markers with respect to the other RFLP markers previously mapped on the same population.

Discussion

Isolation and characterization of sorghum SSR loci

In accordance with previous observations in different plant genomes (Lagercrantz et al. 1993; Morgante and Olivieri 1993), Southern-blot analyses using sorghum genomic DNA digested with different 4- and 6-cutter restriction enzymes (data not shown) demonstrated that $(AG)_n$ repeats are more abundant and dispersed in this genome than are $(AC)_n$ repeats. We cannot provide any estimate on the frequency of the AG repeat, because our SSRs were isolated from a library highly enriched for this repeat. Recent studies in maize (Taramino and Tingey 1996) have demonstrated that $(AC)_n$ repeats are less polymorphic and more difficult to type. One possible explanation for this is the fact that $(AC)_n$ repeats are prevalently found within the class of imperfect repeats, with few bases interrupting the run of the tandem dinucleotide array (Weber 1990). This information, along with the results of our Southern-blotting experiments, induced us to search for $(AG)_n$ repeats.

Our newly isolated sorghum SSRs are characterized by long stretches of AG, with an average of 34 repeat units. However, this result cannot be taken as truly representative of the $(AG)_n$ composition of the sorghum genome, due to the hybridization conditions used during the library enrichment which tends to favor the isolation of clones with long repeats, and due to the relatively small sample size. On the other hand, shorter repeats were found through searches of sorghum sequences deposited in databases. As expected for genetic coding reasons, only trinucleotide repeats [$(GCG)_8$ for SVHRGP and $(ACA)_9$ for SbKAFGK1)] were found within coding sequences. Thus Wang et al. (1994) found that as much as 57% of SSR-containing triplets rich in G-C basepairs were located in coding regions.

Our set of 13 markers (Table 2) was selected for the good amplification pattern obtained using genomic sorghum DNA as a template, and was used for the polymorphism analysis in nine sorghum inbred lines. Although limited, this number of genotypes represents a good sample of the sorghum germ plasm, due to their clear differentiation with regard to geographic origin, morphological characteristics and agronomic traits.

One of the most important parameters of a given marker is the degree of polymorphism, which, in the

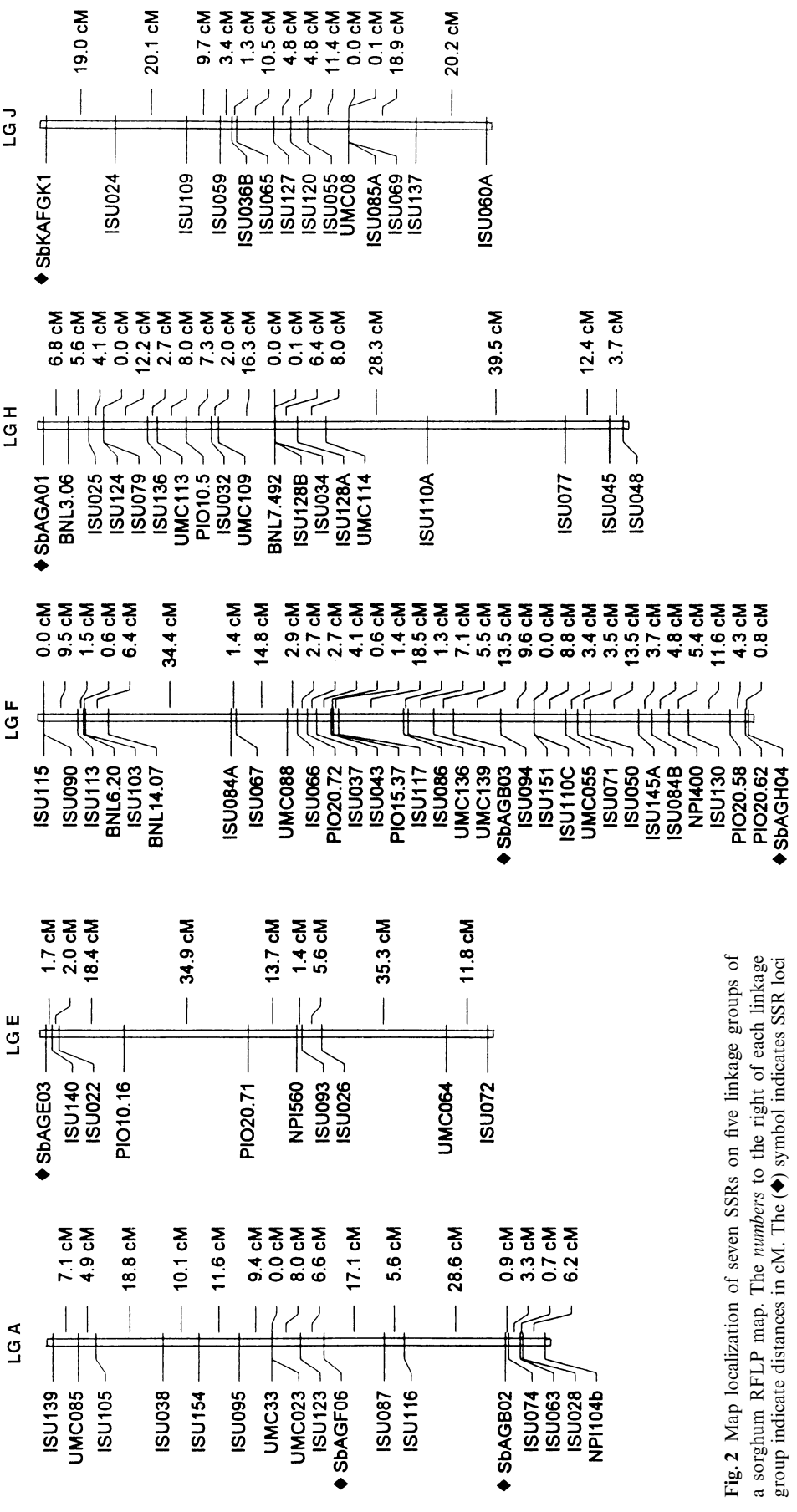

Fig. 2 Map localization of seven SSRs on five linkage groups of a sorghum RFLP map. The *numbers* to the right of each linkage group indicate distances in cM. The (◆) symbol indicates SSR loci

case of SSRs, seems to increase with an increasing number of repeat units (Weber 1990). It is interesting, but not surprising, considering the length of the AG repeats of our SSRs, that none of the SSRs analyzed proved to be monomorphic, depicting a number of different alleles ranging from a minimum of three to a maximum of nine. The informativeness of our SSRs, i.e. the ability of these markers to distinguish between genotypes, was measured in terms of δ_T. This provides a measure of the level of population differentiation and represents the probability with which two members of a population, sampled at random and without replacement, differ in allele composition (Gregorius 1987). This is equivalent to the expected heterozygosity H_e (Nei 1987), excluding the correction for small sample size. SbAGE01, which identifies nine alleles, thus discriminating each of the inbreds analyzed, has the maximum δ_T value of 1. With the exception of SbAGE03 ($\delta_T = 0.46$) and SvHPRGPG ($\delta_T = 0.51$), all the other markers have very high δ_T values. It is interesting to note that the lower value is associated with SbAGE03, the only SSR bearing an imperfect repeat. This seems to be in accordance with the hypothesis that slipped-strand mispairing (SSM), proposed as the main mechanism in producing SSR hypervariability (Levinson and Gutman 1987), is less effective in creating variability when molecules with non-complementary bases, due to the presence of imperfect repeats, are involved (Weber 1990).

It is not surprising that the other low δ_T value has been obtained for the SSR found in the coding region of the hydroxyproline-rich glycoprotein (SvHPRGPG), where a higher degree of conservation, relative to non-coding DNA sequences, is predicted. On the other hand, the $(ACA)_9$ repeat found in the coding sequence of the locus SbKAFGK1 was unexpectedly informative ($\delta_T = 0.80$). The presence of about 20 copies of kafirin per haploid sorghum genome has been estimated (De Rose et al. 1989); however, our set of primers gave a single band amplification pattern which allowed us to assign single alleles to each individual. Furthermore, using the SbKAFGK1 primer set, we were able to map this gene on to sorghum linkage group J (Fig. 2). To our knowledge, this is the first indication of a kafirin map position in the sorghum genome. The higher value of δ_T obtained using this SSR could be associated with the higher number of trinucleotide repeat units (9 versus 8).

The general high level of variation of microsatellites between different inbreds of sorghum indicates that this approach could represent a valid contribution to the genetic characterization of *S. bicolor*. To our knowledge, different investigations of the degree of polymorphism in this species have reported discordant data. Being a self-pollinating crop, it is expected to observe less diversity compared to an out-crossing species, and this expectation has been confirmed by using either RFLP or RAPD markers (Tarchini et al. personal communication; Tao et al. 1993); nevertheless, the relatively good rate of polymorphism found with both isozyme and RFLP studies (Aldrich et al. 1992; Cui et al. 1995) has been ascribed to the multiple origins for domesticated sorghum, cross pollination between races, and a certain level of out-crossing (Dogget 1988). Our data, based on a polymorphism assay which is not dependent on the frequency of point mutations or insertion/deletions at the DNA level, allowed to depict a considerable amount of polymorphism within *S. bicolor*, comparable to that of highly polymorphic species such as maize or potato.

Seven SSR markers were clearly positioned on the linkage groups shown in Fig. 2 with a LOD score greater than 3. The use of this new kind of molecular marker would certainly reduce the number of gaps in the existing sorghum maps and would consequently facilitate sorghum molecular breeding practices.

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