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Genetic diversity of stay-green sorghums and their derivatives revealed by microsatellites

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The genetic variability of 28 sorghum genotypes of known senescence phenotype was investigated using 66 SSR markers well-distributed across the sorghum genome. The genotypes of a number of lines from breeding programmes for stay-green were also determined. This included lines selected phenotypically for stay-green and also RSG 03123, a marker-assisted backcross progeny of R16 (recurrent parent) and B35 (stay-green donor). A total of 419 alleles were detected with a mean of 6.2 per locus. The number of alleles ranged from one for Xtxp94 to 14 for Xtxp88. Chromosome SBI-10 had the highest mean number of alleles (8.33), while SBI-05 had the lowest (4.17). The PIC values obtained ranged from zero to 0.89 in Xtxp94 and Xtxp88, respectively, with a mean of 0.68. On a chromosome basis, mean PIC values were highest in SBI-10 (0.81) and lowest in SBI-05 (0.53). Most of the alleles from B35 in RSG 03123 were found on chromosomes SBI-01, SBI-02 and SBI-03, confirming the successful introgression of quantitative trait loci associated with stay-green from B35 into the senescent background R16. However, the alternative stay-green genetic sources were found to be distinct based on either all the SSRs employed or using only those associated with the stay-green trait in B35. Therefore, the physiological and biochemical basis of each stay-green source should be evaluated in order to enhance the understanding of the functioning of the trait in the various backgrounds. These genetic sources of stay-green could provide a valuable resource for improving this trait in sorghum breeding programmes.

Keywords: Simple sequence repeats, sorghum, stay-green, genetic diversity.

INTRODUCTION

The stay-green trait has been used for years by breeders as a measure of post-flowering drought tolerance (Rosenow and Clark, 1981; Borrell et al., 2001) and is a mechanism that prevents premature senescence under

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low soil moisture stress during grain-filling (Sanchez et al., 2002). The trait is characterised by green stems and green upper leaves when water is limiting during grain filling, which enables the stay-green genotypes to continue to fill their grain under drought conditions (Subudhi et al., 2000; Tao et al., 2000; Borrell et al., 2000 a, b, Borrell et al., 2001). Understanding the inheritance of traits, such as stay-green, is important for its successful application in plant breeding programmes. Markers associated with regions of the genome controlling stay-green can be used in the breeding programme to assist in the precise introgression of those regions into novel varieties. Alleles of simple sequence repeats (SSR) associated with stay-green quantitative trait loci (QTL) have been identified in a number of genetic sources (Borrell et al., 2000ab; Bhattramakki et al., 2000; Kebede et al., 2001; Kong et al., 2000; Haussmann et al., 2002), which might influence their tolerance on post-flowering drought stress. However, different genetic sources of stay-green may employ different genes and display different inheritance characteristics.

Analysis of genetic diversity is important for crop improvement and provides essential information to enable more efficient use of available genetic resources and a platform for stratified sampling of breeding populations (Mohammadi and Prasanna, 2003). Accurate assessment of the levels and patterns of diversity can be invaluable in the analysis of genetic variability in cultivars (Smith, 1984; Cox et al., 1986), identification of diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998) and in introgressing desirable genes from diverse germplasm into the available genetic base (Thompson et al., 1998). Genetic relationships can be useful for the planning of crosses, assigning lines to specific heterotic groups and for precise identification with respect to plant varietal protection (Mohammadi and Prasanna, 2003).

Molecular markers have many advantages over phenotypic characters as they are unaffected by the environment. DNA-based markers, such as simple-sequence repeats (SSRs), have been or are being utilised in cultivar development, quality control of seed production, measurement of genetic diversity for conservation management, varietal identification and intellectual property protection (Smith et al., 2000; McIntyre et al., 2001) and are powerful tools in genetic similarity studies (Pejic et al., 1998). Furthermore, these markers can be used to obtain information on the genes that influence agriculturally important traits and to follow the introgression of these genes, thus facilitating the breeding process.

SSRs are highly useful as genetic markers as they are codominant, occur in high frequency and appear to be distributed well throughout the genomes of higher plants and animals (Bhattramakki et al., 2000) with simple Mendelian segregation (Brown et al., 1996). SSRs have higher information content, as measured by expected heterozygosity and number of alleles and polymorphism, as compared to amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPDs) and restriction fragment length polymorphism (RFLPs) (Smith et al., 1997; Pejic et al., 1998; Uptmoor et al., 2003), indicating that SSRs are more discriminating. The high information content and favourable characteristics of SSRs make them excellent genetic markers for many types of investigations, including marker-assisted selection (MAS) and finger-printing of germplasm collections (Kong et al., 2000). Comparative studies in crop plants have shown that they provide a powerful tool for discriminating between genotypes and for MAS (Djè et al., 2000; Asare et al., 2010; Asare et al., 2011; Faustine et al., 2015).

In sorghum, SSRs are well distributed across the sorghum genome (Kong et al., 2000). It is thus possible to avoid overrepresentation of certain regions of the genetic map that can produce inaccurate estimates of genetic similarities among individuals. SSRs have revealed high levels of polymorphism to allow the vast majority of *Sorghum bicolor* accessions, including those within working groups, to be distinguished by using a relatively small number of SSR loci (Taramino et al., 1997; Kong et al., 2000 Smith et al., 2000; Xu et al., 2000; Uptmoor et al., 2003; Menz et al., 2004). Indeed, 10 SSRs were able to distinguish 324 individuals across 27 sorghum accessions (Ng'uni et al., 2011). In addition to their use in genetic diversity analysis, SSRs have been widely used in genetic mapping and QTL analysis in sorghum.

For the study of stay-green, the line B35 or its derivative QL41 has been employed as one of the parents in the development of mapping populations (Tuinstra et al., 1997; Crasta et al., 1999; Subudhi et al., 2000; Tao et al., 2000; Xu et al., 2000) and some of the QTL identified (stg1, stg2, stg3 and stg4) from B35 have been introgressed into the senescent R16 background using marker-assisted backcrossing (Hash et al., 2003, Harris et al., 2007; Kassahun et al., 2010; Vadez et al., 2011). Relatively, few studies have used different stay-green sources, such as SC56 (Kebede et al., 2001) and E36-1 (Haussmann et al., 2002). Although, the B35 and KS19 sources of stay-green have been employed in breeding programmes in the USA and Australia (Borrell et al., 2000a, b) little is known about the genetic basis of the stay-green trait in KS19. There may be other sources of stay-green genotypes yet untapped. It is important that breeding programmes should not rely on one or a few sources of any trait in order to reduce the risk of genetic vulnerability. Furthermore, the genetic and physiological determinants of the trait are varied (Thomas and Howarth, 2000). Therefore, it would be good to widen the base of the stay-green sources to avoid any future vulnerability yet unknown. In addition, as shown in studies by Borrell et al. (2000a, b) and Haussmann et al.

Table 1. Races, origins and senescence phenotypes of sorghum genotypes investigated.

Line	Race, country of origin, and breeding programmes	Senescence phenotype
2077-B	Durra, breeding line from India	Senescent
296B	Breeders' material from India	Stay-green
B35	Bred in Texas from a Durra line originally from Ethiopia. Employed in breeding programmes in Australia and the USA. Also referred to as BTx642	Stay-green
BTx623	Kafir x Zera-zera, Texas A & M, source 90C510	Senescent
DJ1195	Durra, breeding line from India	Senescent
E36-1	Widely-adapted zere-zera line from Ethiopia; high-yielding, Guinea-caudatum	Stay-green
#66	Guinea-caudatum, derived from a cross between E36-1 and R16 from ICRISAT	Stay-green
#68	Guinea-caudatum, derived from a cross between E36-1 and R16 from ICRISAT	Stay-green
ICSV112	Guinea-caudatum, bred at ICRISAT Centre and originally released as a variety in Zimbabwe	Stay-green
IS13441	Caudatum, landrace from Zimbabwe	Senescent
IS18530	Durra, breeding material from Egypt	Senescent
IS18551	Durra, breeding material from Ethiopia	Stay-green
IS2146	Durra, landrace from Nigeria	Senescent
IS22830	Caudatum, landrace from Sudan	Stay-green
IS3260C	Guinea/Margaritiferum, landrace from Nigeria	Stay-green
IS3762	Bicolor, landrace from China	Senescent
IS4845	Durra, landrace from India	Senescent
IS9302	Kafir, advanced cultivar from South Africa	Stay-green
IS9830	Caudatum, advanced cultivar from Sudan	Senescent
KS19	Derived from a cross between a Short Kaura (a landrace from Nigeria) and Combined Kafir 60. Used for breeding in Australia and the USA for the stay-green trait.	Stay-green
QL12	Dwarf genotype with a KS19 pedigree bred in Australia	Stay-green
QL39	Dwarf genotype with SC170C and KS4 pedigree bred in Australia	Senescent
QL41	A hybrid from a cross between B35 and QL33 bred in Australia	Stay-green
R16	High-yielding post-rainy season variety from Maharashta; a Guinea-caudatum of Ethiopian origin	Senescent
R9188	Texas A & M, Source 90C66	Stay-green
RSG 03123	A BC_1F_5 R16 introgression line with *Stg 1, 2, 3, and 4* QTL from B35 developed at ICRISAT	Stay-green
SC56	Caudatum-nigricans, breeding material from Sudan	Stay-green
SPV386	Caudatum from India	Senescent

(2002), the senescent parents of the mapping populations used (QL39 and N13) contributed major QTL for stay-green to the hybrids. Therefore, this study was designed to examine the genetic variability among sorghum germplasm normally grown in the semi-arid regions, determine the genetic variability using SSRs associated with the stay-green trait in B35 and to assess the genotypes of stay-green derivatives of B35, KS19 and E36-1 sources of stay-green which have been developed either using phenotypic or marker-assisted selection. The stay-green trait, as a post-flowering drought resistance mechanism, has great potential for use in breeding and selection of sorghum lines for drought-prone agro-ecological zones. Therefore, such studies should enhance our understanding of the genetic relatedness of sorghum germplasm and enhance breeding for elite cultivars of sorghum for the semi-arid and arid regions.

MATERIALS AND METHODS

Plant material and DNA extraction

The races, origins and senescence phenotypes of the sorghum genotypes employed in the study are described in Table 1. Leaves were harvested at the 3-4 leaf stage from 20 seedlings of each line and stored at -80°C prior to DNA extraction using the Nucleon PhytoPure Plant and Fungal DNA Extraction Kit RPN 8511 (Amersham Life Science, Little Chalfont, England) according to the manufacturer's instructions. DNA was quantified using a UV-VIS PU8720 spectrophotometer (Philips) and its integrity checked by running on 1% agarose gels stained with ethidium bromide. Each well contained a mixture of loading buffer (5 µl) and sample DNA

(10 µl). The gels were run with 1x TAE buffer from the cathode to the anode with a constant voltage of 70mV for 1.5 h. DNA samples were diluted with sterile nano-pure water to a final concentration of 10 ng/µl.

PCR amplification of SSRs

Seventy-four SSR primer pairs were chosen by initially selecting at least five SSR loci from each of the 10 sorghum genetic chromosomes to uniformly represent the entire mapped nuclear genome (Bhattramakki et al., 2000; Kong et al, 2000). Of these, only sixty-eight displayed polymorphism in a preliminary study and these were used with all genotypes tested (Table 2). PCR reactions were conducted in GeneAmp® PCR System 2700 (Applied BioSystems) in a 25 µl reaction mixture in 96-well plates. The 25-µl reaction mix consisted of 1× PCR buffer, 50 ng genomic DNA template, 4 µl of dNTP (5 mM equimolar solution of each dATP, dCTP, dGTP and dTTP), 1.25 µl each of forward and reverse primers (10 µM solution) and 0.65 U *Taq* DNA polymerase (Roche Applied Sciences). The PCR programme consisted of an initial denaturation for 2 min at 94°C and then 30 cycles of denaturation for 30 s at 94°C, primer specific annealing for 30 s (Table 2) and extension at 72°C for 90 s. After 30 cycles, there was a final extension step of 7 min at 72°C. Amplification products were separated on a 4.5% polyacrylamide, 8.5 M Urea denaturing gel (BIO-RAD Sequi-GenGT) and visualised by silver staining (Promega Silver Sequencing system) along with a 50 bp ladder (Gibco).

Analysis of data, genotyping and determination of genetic diversity

Bands for the same SSR locus with different molecular weights were scored as alleles. Presence or absence of each amplified band was scored as 1 and 0, respectively, for all markers to generate a binary data matrix. The genetic diversity for each microsatellite locus was calculated according to the following formula of Nei (1973):

Genetic diversity = $1- \Sigma P_{ij}^2$,

where P_{ij} is the frequency of *jth* allele for the *ith* locus summed across all the alleles of the locus. Calculated in this manner, the genetic diversity is synonymous with the term polymorphic information content (PIC) described by Anderson et al. (1993).
The binary data matrix generated from this scoring was used to calculate a similarity matrix using the Nei and Li (1979) coefficient. Cluster analysis was conducted using the unweighted paired group method using arithmetic averages (UPGMA) as defined by Sneath and Sokal (1973) to produce dendrograms of genetic similarities using the Numerical Taxonomy and Multivariate Analysis System software (NTSTSpc) version 2.1 (Exeter Software, New York). The correlation coefficient between the similarity matrix and the cophenetic values matrix was computed to test the goodness of fit of the cluster analysis. The binary data matrix was also analysed using only the data for 15 loci (as highlighted in Table 2) associated with the published B35 QTL for stay-green.

RESULTS

Size of SSRs, number of alleles and polymorphism information content (PIC)

The estimated sizes of SSR alleles, number of alleles produced and the PIC values of the 68 SSR loci examined are presented in Table 2. A total of 419 alleles were detected among the 28 genotypes assessed. The number of alleles per locus ranged from one (*Xtxp*94) to 14 (*Xtxp*88) with an overall mean of 6.2. Thus, only one locus (*Xtxp*94) was non-polymorphic. This marker was included in an attempt to increase genome coverage on chromosome SBI-05. SBI-02 and SBI-03 had the highest number of loci, while SBI-10 had the smallest number. The mean number of alleles was highest in SBI-10 (8.3) followed by SBI-09 and SBI-06 (7), SBI-01 and SBI-04 (6.8), SBI-02 (6.6) SBI-08 (6.4) SBI-07 (5.5), SBI-03 (5.3) and SBI-05 with the least (3.7). These markers had all been assessed previously in a set of 18 relatively diverse sorghum genotypes (Kong et al., 2000; Bhattramakki et al., 2000). In the set of 28 genotypes examined in this study, 49 (72%) loci had, at least, one allele more than previously reported, while 10 (15%) loci had at least one less allele and 9 (13%) loci had the same number. The mean number of alleles for SSR loci associated with the stay-green trait in the various published studies was 6.2, which was the same for all 68 SSR loci. Loci *Xtxp*88, *Xtxp*285, *Xtxp*56, *Xtxp*298, *Xtxp*207, *Xtxp*230, *Xtxp*217 and *Xtx*p67 associated with the stay-green trait were the most polymorphic with alleles varying from 14 in *Xtxp*88 to 7 in *Xtxp*67.

PIC values ranged from zero (*Xtxp*94) to 0.89 (*Xtxp*88 and *Xtxp*285) with a mean of 0.68. PIC values for these loci have not been reported before. *Xtxp*88 in chromosome SBI-01 had 14 alleles (highest), while *Xtxp*285 had 12 in chromosome SBI-03 (second highest). Chromosome SBI-10 again had the highest mean PIC value of 0.81. This was followed by chromosome SBI-06 (LG I) (0.78), SBI-04 (0.74), SBI-09 and SBI-08 (0.71), SBI-02 (0.70), SBI-01 (0.68), SBI-03 (0.64), SBI-07 (0.60) and SBI-05 again with the lowest PIC value of 0.53. The proportion of loci with PIC values equal to or more than 0.5 was 85%, while 82% had PIC values from 0.6 and PIC values in 69% were equal to or more than 0.7. The mean PIC value for SSR loci associated with the stay-green trait was 0.64, lower than the overall mean.

Genetic diversity among the 28 sorghum genotypes

PCR products from these 68 SSR primers were used to evaluate genetic diversity in 28 sorghum lines. Associations between the lines studied based on the cluster analysis of their genetic similarities are presented in a dendrogram (Figure 1). The set of markers used was able to uniquely classify the 28 lines included in this study and showed that considerable genetic diversity was present. Five distinct groups were identified in the resulting dendrogram. The most genetically distinct genotype of those examined was IS3620C, which did not cluster with any other line. This is the only Guinea-Margaritiferum line included in the study and was included (along with BTx623) as it is a parent in the

Table 2. Size, number of alleles and polymorphic information content (PIC) of SSR markers used in genetic diversity analyses.

Locus	[1]Chromosome no.	Annealing temperature (°C)	[2]No. of alleles	Size range in this study		No. of alleles in this study	PIC in this study
Xtxp58	SBI-01	55	7	145	161	6	0.79
Xtxp61	SBI-01	55	3	121	190	8	0.80
Xtxp88	SBI-01	53	6	106	150	14	0.89
Xtxp208	SBI-01	55	3	227	231	2	0.44
Xtxp229	SBI-01	55	2	153	157	3	0.43
Xtxp316	SBI-01	55	6	279	362	9	0.75
Xtxp335	SBI-01	55	5	133	220	9	0.80
Xtxp357	SBI-01	55	3	238	244	3	0.57
Xtxp8	SBI-02	60	6	115	155	9	0.84
Xtxp56	SBI-02	55	5	268	497	10	0.82
Xtxp96	SBI-02	52	5	140	174	7	0.77
Xtxp100	SBI-02	55	2	125	127	2	0.33
Xtxp207	SBI-02	55	4	149	172	5	0.79
Xtxp211	SBI-02	55	6	180	250	10	0.91
Xtxp283	SBI-02	55	6	203	205	2	0.32
Xtxp286	SBI-02	55	2	179	201	4	0.57
Xtxp296	SBI-02	55	5	165	177	5	0.70
Xtxp298	SBI-02	55	5	151	196	10	0.86
Xtxp348	SBI-02	55	4	226	350	9	0.80
Xtxp38	SBI-03	60	4	406	446	5	0.72
Xtxp69	SBI-03	50	4	186	270	9	0.84
Xtxp114	SBI-03	50	2	200	208	3	0.43
Xtxp183	SBI-03	55	3	165	170	3	0.65
Xtxp205	SBI-03	55	4	198	210	6	0.69
Xtxp215	SBI-03	50	3	172	182	4	0.66
Xtxp218	SBI-03	55	2	190	260	5	0.55
Xtxp228	SBI-03	55	4	205	228	4	0.66
Xtxp231	SBI-03	55	2	188	210	3	0.45
Xtxp285	SBI-03	55	5	215	258	12	0.89
Xtxp336	SBI-03	55	3	146	154	4	0.56
Xtxp12	SBI-04	55	4	164	210	6	0.77
Xtxp21	SBI-04	60	5	145	163	7	0.72
Xtxp177	SBI-04	55	4	150	160	4	0.61
Xtxp343	SBI-04	55	5	120	220	10	0.84
Xtxp94	SBI-05	50	2	220	220	1	0.00
Xtxp30	SBI-05	60	7	147	161	4	0.72

[1]Chromosome number based on Bhattramakki et al. (2000) as published by Kim et al. (2005); [2]Number of alleles detected among 18 diverse sorghum strains listed in Table 1 of Kong et al. (2000). SSRs markers associated with the stay-green QTL in B35 are highlighted (bold).

production of the most complete microsatellite map of sorghum (Bhattramakki et al., 2000) on which all the markers used here are mapped. This line also was the most genetically distinct in the study of Menz et al. (2004).

The largest group consisted of twelve genotypes with B35 at one end and IS9302 at the other. This group was subdivided into five, with the first subgroup containing B35 and QL41. QL41 is an inbred line derived from a cross in which B35 was a parent. The second subgroup contained KS19 and QL12, which had many alleles in common. Indeed these two lines were the most similar of

Table 2. Contd.

Locus	[1]Chromosome no.	Annealing temperature	[2]No. of Alleles	Size range in this study		No. of alleles in this study	PIC in this study
Xtxp115	SBI-05	60	2	201	207	3	0.56
Xtxp299	SBI-05	50	5	190	212	5	0.69
Xtxp123	SBI-05	55	ND	260	280	3	0.56
Xtxp136	SBI-05	55	ND	221	229	3	0.36
Xtxp225	SBI-05	55	4	159	178	7	0.80
Xtxp6	SBI-06	50	9	340	398	5	0.77
Xtxp17	SBI-06	55	3	148	177	7	0.81
Xtxp57	SBI-06	55	3	240	252	6	0.75
Xtxp145	SBI-06	55	5	184	210	8	0.77
Xtxp265	SBI-06	55	8	160	199	11	0.82
Xtxp274	SBI-06	55	6	305	350	5	0.77
Xtxp40	SBI-07	55	2	124	134	4	0.52
Xtxp92	SBI-07	50	2	148	162	2	0.32
Xtxp168	SBI-07	55	3	158	164	4	0.69
Xtxp278	SBI-07	50	3	246	254	3	0.43
Xtxp295	SBI-07	55	5	134	162	10	0.78
Xtxp312	SBI-07	55	9	130	194	10	0.85
Xtxp47	SBI-08	55	3	229	236	4	0.66
Xtxp105	SBI-08	55	3	265	274	4	0.60
Xtxp210	SBI-08	55	4	167	190	8	0.70
Xtxp273	SBI-08	55	5	180	207	7	0.76
Xtxp321	SBI-08	55	4	170	230	9	0.83
Xtxp67	SBI-09	55	8	145	172	7	0.83
Xtxp230	SBI-09	55	10	220	270	8	0.83
Xtxp258	SBI-09	55	5	162	199	8	0.77
Xtxp287	SBI-09	55	5	345	353	3	0.59
Xtxp289	SBI-09	55	5	227	282	10	0.84
Xtxp339	SBI-09	55	2	150	186	4	0.20
Xtxp358	SBI-09	55	4	220	299	9	0.88
Xtxp141	SBI-10	55	5	126	150	8	0.78
Xtxp217	SBI-10	55	5	152	168	8	0.82
Xtxp270	SBI-10	55	6	208	273	9	0.84

[1]Chromosome number based on Bhattramakki et al. (2000) as published by Kim et al. (2005); [2]Number of alleles detected among 18 diverse sorghum strains listed in Table 1 of Kong et al. (2000). SSRs markers associated with the stay-green QTL in B35 are highlighted (bold).

any of the genotypes examined. BTx623 also clustered with KS19 and QL12. The third sub-group included seven genotypes, but there was considerable diversity between these lines. R9188, which is a stay-green line from the Rio source of stay-green (Borrell, personal communication), was found in this sub-cluster as was QL39, the senescent parent used in the QTL analysis of Tao et al. (2000). This study found that unexpectedly, QL39 contributed a number of stay-green alleles. The next largest group comprised of nine genotypes with E36-1 and #68 at one end and IS18530 at the other. The group had four subgroups; the first included E36-1 and the 2 lines derived from it #66 and #68. The second subgroup consisted of IS22380, DJ1195 and IS13441. The third subgroup had ISCV112 and SPV386 with IS18530 alone in the fourth subgroup. E36-1, IS22380, IS13441, ICSV112 and SPV386 are all caudatum type sorghums. Clustering however did not always follow the sorghum race classification (or country of origin) particularly as a number of the advanced breeding lines

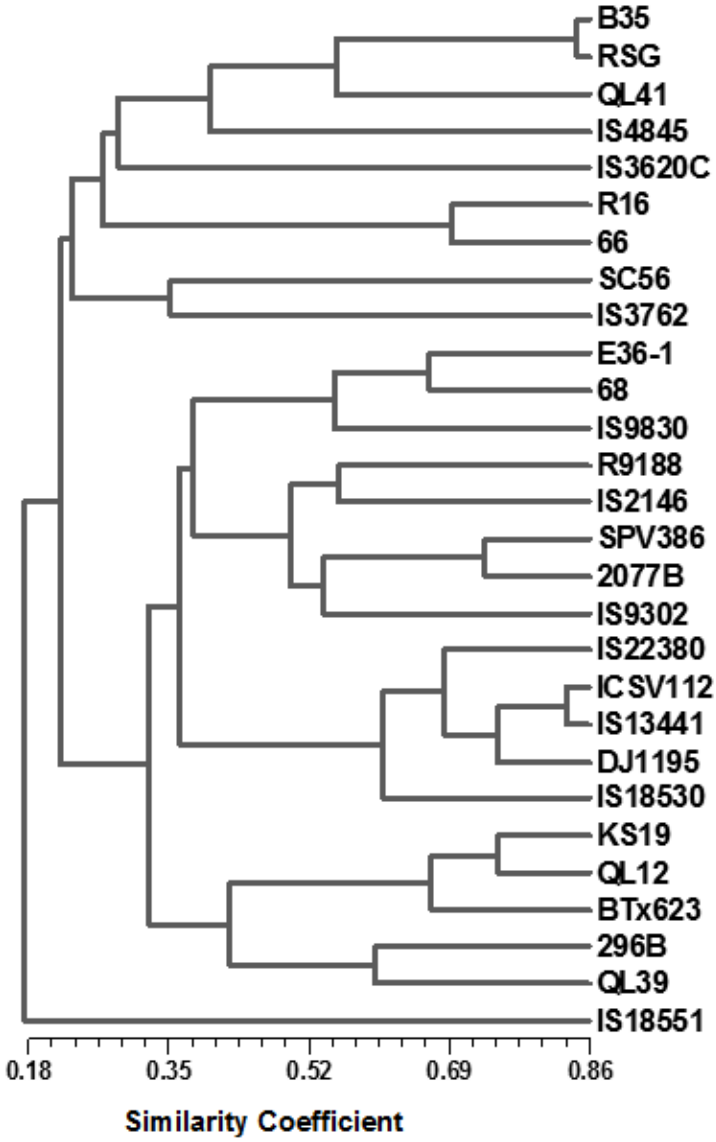

Figure 1. Dendrogram constructed with unweighted paired group method using the arithmetic average (UPGMA) clustering algorithm from pairwise matrix of genetic similarities of 28 sorghum lines using 68 microsatellite markers.

examined have more than one race in their genetic background. This has also been found in other studies of genetic diversity in sorghum (Menz et al., 2004).

The fourth major group consisted of four genotypes, with R16 and RSG 03123 in a subgroup, and IS18551 and IS4845 in another subgroup. R16 and RSG 03123 are guinea-caudatum race sorghums, but RSG 03123 has genes from the Durra sorghum B35; IS18551 and IS4845 are Durra race sorghums. The fifth major group had only two genotypes SC56 and IS9830, which both originate from Sudan and were about 40% similar.

Genetic similarities based on SSRs associated with the B35 stay-green QTL only

A dendrogram of genetic similarity based on 15 SSRs associated with the four major B35 stay-green QTL identified three major groups (Figure 2). The first distinct group had IS3762 and B35 at either end, with three subgroups. The largest subgroup comprised B35, RSG 03123, QL41, IS4845 and IS3620C. B35 and RSG 03123 were most related with a coefficient of more than 0.8 and were more similar to QL41 than the other genotypes. This

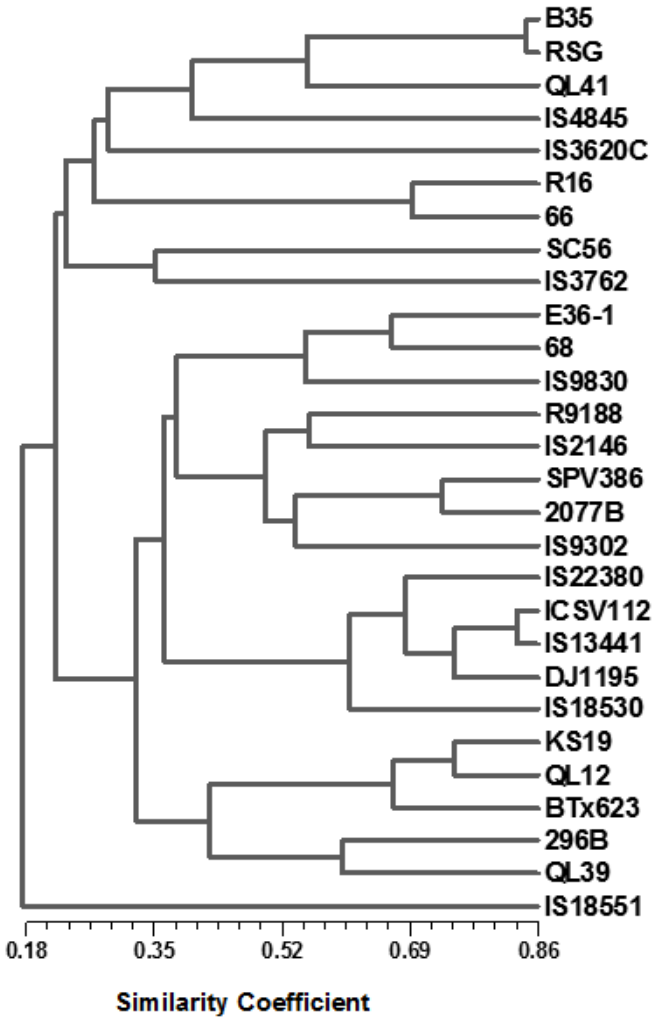

Figure 2. Genetic similarity among sorghum cultivars based on 15 SSRs associated with the B35 stay-green QTL only using UPGMA.

was not surprising as RSG 03123 was developed by marker-assisted backcrossing of B35 alleles associated with the stay-green QTL. The second subgroup had R16 and #66, while SC56 and IS3762 were in the third. The stay-green line SC56 was much more genetically similar to B35 when only regions of the genome comprising B35 stay-green QTL were considered. Kebede et al. (2001) found that a number of stay-green QTL were common between SC56 and B35, suggesting that common genes were involved in these otherwise genetically distinct lines. The second distinct group had 13 genotypes with IS18530 and E36-1 at each end. This indicates that, as with the overall genetic diversity, E36-1 shares little in common with B35 at the B35 stay-green regions. The stay-green line R9188 also clustered in this group as did IS22380. This latter line was found to have a high expression of stay-green in the study of Mahalakshmi and Bidinger (2002). The third major group included five genotypes, with KS19, QL12 and BTx623 in one subgroup, as well as 296B and QL39 in another. KS19 and QL12 were much more similar to B35 when their overall genetic similarity was examined. IS18551 which fell into the same group with IS4845 in Figure 1, this time stood alone with only 18% similarity with all other genotypes.

Genotypes of derivatives in relation to the parental lines

Five of the accessions studied (RSG 03123, #66, #68, QL12 and QL41) are known to be derived from accessions also included in this study. RSG 03123 is the product of marker-assisted backcrossing (MABC)

conducted at ICRISAT in which the target was to transfer genomic regions on chromosomes BSI-01, SBI-02, SBI-03 and SBI-05 from B35 where stay-green QTL have been identified in a wide range of studies (Kong et al., 2000; Bhattramakki et al., 2000; Huassmann et al., 2002).

For 31 loci, RSG 03123 was homozygous for the R16 parental allele whereas for 18 loci it was homozygous for the B35 allele. Ten SSR loci were non-polymorphic, one was heterozygous and 3 loci did not produce PCR products. Most of the alleles from B35 were found on chromosomes SBI-01, SBI-02 and SBI-03. Out of nine loci on chromosome SBI-01, alleles of four SSRs came from each parent while the remaining one was not polymorphic. The alleles from R16, however, were associated with the position of the B35 QTL stgA indicating that this QTL was not transferred from B35 into R16. In chromosome SBI-02 one locus was heterozygous for both parents; six of the rest were from B35 and three from R16. All the SSRs associated with *stg*3 from B35 were present in RSG 03123, while the SSRs associated with stgB originated from R16. In chromosome SBI-03, four SSRs were from B35; three of them are associated with *stg*1, while three SSRs were of R16 origin. In the genomic region where stg2 is located, there was no polymorphism detected in the markers used between B35 and R16. Chromosomes SBI-04, SBI-06, SBI-08 and SBI-09 were entirely made up of alleles from R16. In chromosomes SBI-05, SBI-07 and SBI-10, one allele from B35 was found in each of them. stg4 is in chromosome SBI05 and although for one marker associated with this QTL, the B35 allele was found in RSG 03123, again lack of polymorphism meant that it was not possible to determine whether this QTL had been transferred from B35 to RSG 03123 or not.

Two regions of the genome corresponding to QTL for stay-green (stg1 and stg3) were homozygous for B35 alleles confirming that stg1 and stg3 were successfully transferred from B35 to R16. The non-polymorphism between R16 and B35 in the other major regions containing stay-green QTL makes it impossible to confirm the transfer of *stg*2 and *stg*4 from B35 to R16. The minor QTL from B35 (stgA and stgB) would appear not to have been transferred to RSG 03123 as its genomic composition in these regions were homozygous for the R16 allele. As the majority of the genome of RSG 03123 is R16, these results indicate that this material is ideal for the study of the functioning of stay-green QTL in a novel or senescent background.

Genotypes #66 and #68 are phenotypic selections from a cross between E36-1 and R16. E36-1 is described as a stay-green line (van Oosterom et al., 1996) although its stay-green nature is dependent on the environment in which it is grown. In the phenotypic study of Mahalakshmi and Bidinger (2002), E36-1 was not described as stay-green, but it was used as the stay-green parent in both (2) populations used in the QTL analysis of stay-green of Haussmann et al. (2002). QTL in which E36-1 provided the positive alleles for stay-green were found on chromosomes SBI-01, SBI-03, SBI-07 SBI-08 and SBI-10. The hybrids #66 and #68 showed a different stay-green response in the field study of Mahalakshmi and Bidinger (2002) but no genetic analyses have been conducted previously with this material. #66 and #68 had the same alleles at 22 SSR loci. They both shared four alleles with R16 and 14 alleles with E36-1. However, #66 had 21 SSR alleles in which the parental allele came from E36-1 and 26 from R16, while in #68 35 SSR alleles originated from E36-1 and only 9 originated from R16. On chromosome SBI-10, #66 and #68 were 100% similar with all SSR alleles being of E36-1 origin. These alleles are also associated with the stay-green QTL from E36-1. Other SSR alleles associated with stay-green QTL from E36-1 on chromosomes SBI-01, SBI-07 and SBI-08 were found in #68 whereas #66 only contained stay-green alleles from E36-1 on SBI-03 in addition to those on SBI-10. QL12 is a sugarcane mosaic virus resistant BC_1 derivative of the stay-green line KS19 (Jordan et al., 2004). It was thus interesting to examine the genomic composition of QL12 as compared to KS19. Only six SSR alleles identified were unique to QL12; three of them occurred in chromosome SBI-03 with one each in SBI-02, SBI-07 and SBI-08. On chromosomes SBI-01, SBI-04, SBI-05, SBI-06, SBI-09 and SBI-10, all the SSR alleles on QL12 were common to KS19 and in chromosome SBI-02, nine out 10 SSRs were also observed in KS19. Neither KS19 nor QL12 have been included in any published mapping studies for stay-green, so it is not known, which regions of the genome control stay-green expression in these genotypes or whether there is any similarity to those found for other stay-green lines such as B35.

QL41 is a stay-green line derived from a cross between B35 and QL33 (Jordan et al., 2004) and has been used as a parent to identify QTL associated with stay-green in a cross with QL39 (Tao et al., 2000). Twelve SSR alleles were unique to QL41 and another 11 were common to B35 and QL41 as well as many other cultivars. SSR alleles from B35 were found in SBI-02 and these were associated with the stay-green QTL mapped in QL41 as well as stg3 and stgB in B35 suggesting that this stay-green QTL in QL41 originates from B35. QL41 did not have the same alleles as B35 at the position of the other B35 stay-green QTL. Furthermore, there were 13 SSR alleles found in QL41, which were common to KS19 but not B35.

DISCUSSION

Scoring of SSRs

The 68 SSR markers used in this study generated 416

alleles with 1 to 14 alleles per locus with an average of 6.2. Similarly, Shehzad et al. (2009) used 38 polymorphic SSR markers to generate 146 alleles which were able to uniquely classify 320 sorghum accessions of diverse origin and the number of alleles revealed per locus ranged from two to nine with average of 3.84. A study of 27 sorghum accessions from a gene bank in Zambia using only 10 SSR loci found a total of 44 alleles from 324 individuals across accessions, a range of 2 to 9 alleles per locus and an average of 4.4 alleles per locus. In the current study, average number of alleles per locus was higher than that in the studies of Shehzad et al. (2009) and Ng'uni et al. (2011).

SSRs are highly polymorphic and useful genetic markers that have been used in genetic similarities studies in wheat (Plaschke et al., 1995; Röder et al., 1995), maize (Smith et al., 1997) as well as sorghum (Taramino et al., 1997; Uptmoor et al., 2003; Menz et al., 2004). The results of Taramino et al. (1997) showed that SSRs have great potential for discriminating among sorghum inbred lines and one SSR locus alone could allow all nine inbred lines to be identified. Polymorphic information content (PIC), a measure of the discrimination ability of a locus, has been found to be comparable between SSRs and RFLPs (Smith et al., 1997) or AFLPs (Menz et al., 2004) or even higher for SSRs (Pejic et al., 1998). The mean PIC and the mean number of alleles per locus for this study were higher than those found by Smith et al. (1997) among 58 maize inbred lines using 131 SSRs and Smith et al. (2000) among 50 elite sorghum lines and similar to that found of Menz et al. (2004). Eight SSR loci with number of alleles from 7 to 14 also had high PIC values (0.79 to 0.89). SSR allele sizes were also similar to those previously reported (Kong et al., 2000; Bhattramakki et al., 2000).

Genetic similarities based on 68 SSRs

All 28 sorghum lines were distinguished and the clusters obtained consisted of the derivatives in close association with their parental lines (Figures 1 and 2). QL41 was in the same cluster with B35, #66 and #68 with E36-1, and RSG 03123 was closely associated with R16. KS19 and QL12 were highly related with a coefficient of more than 0.8, while all other similarities were below this value. In previous studies (Taramino et al., 1997; Smith et al., 2000; Uptmoor et al., 2003; Menz et al., 2004), comparisons were made between R-lines and B-lines or were based on region of origin. The current study was not based on any of these classifications; however, clusters were not always made up of only lines from the same region or race. For example, IS22830 (Sudan, caudatum), DJ1195 (India, Durra), IS13441 (Zimbabwe, caudatum) from different geographical regions or races belonged to the same cluster. Similarly, ICSV112 (India, guinea-caudatum) and SPV386 (India, caudatum) also belonged to same cluster (Figure 1), while IS3620C and KS19 did not cluster together even though both originated from Nigeria. The advantage of using markers with known map positions instead of a random sample is that there is control over the coverage of the genome and although there were a few gaps, the markers used in his study provided extensive marker coverage of the sorghum genome.

Genetic diversity based on SSRs associated with the B35 stay-green QTL

Genetic diversity is vital in the success of any breeding programme (Ali et al., 2007) and molecular markers are an excellent tool for assessment of genetic relationships (Ritter et al., 2007). Initial inheritance studies of the stay-green trait in B35 suggested that it is influenced by a major gene that exhibits varied levels of dominant gene action depending on the environment in which evaluations are made (Tenkouano et al., 1993; Walulu et al., 1994). Subsequent QTL analysis studies indicated at least 4 regions of the genome controlling the trait. A number of clusters were obtained based on differences in allelic sizes of the SSRs associated with the stay-green trait (Figure 2). Derivatives of B35 and KS19 were always in the same clusters with the parental lines, #68 was grouped with E36-1, while #66 was grouped with R16. These associations were consistent with the data information on the data matrix (Table 2). In RSG 03123 stg1 and stg3 were polymorphic between R16 and B35 and RSG 03123 had the same alleles as B35, while stg2 and stg4 were non-polymorphic, hence the close association of RSG 03123 with B35. Similarly, for QL12, apart from a few loci, the SSR alleles were same as in KS19, thus their close relationship. The clustering of #66 with R16 is not surprising since #66 shared 26 SSR alleles of the same size with R16, and 21 with E36-1. #68, which had 35 SSRs of the same size as in E36-1 and only 7 of the same size in R16, was clustered with E36-1, as expected. Panicle shape and size were also similar between E36-1 and #68, and also for #66 and R16 (data not shown). Since R16 has not been involved in any mapping, it is impossible to tell whether it has genomic regions associated with stay-green or not, even though some alleles of SSRs associated with stay-green in B35 were revealed in it.

A mapping population of a cross between B35 and R16 could enhance the understanding of the genetic basis of the trait. KS19 has not been reported as a parent in any mapping population even though it is being used in breeding programmes in the USA and Australia. QTL determined with KS19 as a parent would reveal those contributed by this line and what phenotypic attributes they are associated with.

Conclusions

The 28 sorghum lines were highly variable genetically and clustered into groups not necessarily based on country of origin or race. Derivatives of B35, KS19 and E36-1 clustered together with their parental lines based on all 68 SSRs, while IS3620C was the least related with any other genotype.

Clusters based on the SSRs associated with QTL from B35 alone were similar to those observed with all 68 SSRs for B35, KS19 and E36-1 and their derivatives, except #66 which was in the same cluster with R16 and RSG 03123 clustered with B35. This confirms the introgression of the QTL from B35 into R16. The stay-green line SC56 was much more genetically similar to B35 when only regions of the genome comprising B35 stay-green QTL were considered, suggesting that common genes were involved in these otherwise genetically distinct lines. The stay-green lines KS19, E36-1, R9188 and IS22380 clustered distinctly from B35 indicating that these lines share little in common with B35 at the B35 stay-green regions. This might be so with other sources of stay-green. Hence, there is the need to investigate the physiological and biochemical basis of the trait in these lines and other sources of stay-green as well. These stay-green lines have considerable potential to increase the genetic diversity of the stay-green trait within sorghum breeding programmes. This study also shows how it is possible to follow, through a breeding programme, alleles associated with key agronomic QTL of interest.

Conflict of Interests

The authors have not declared any conflict of interests.

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