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Full Length Research Paper

Comparison between random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers with high resolution melt analyses in genetic variation analysis among selected sorghum genotypes

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Understanding the genetic diversity of germplasm is essential in plant breeding programmes and germplasm management. Molecular markers are efficient and effective tools widely used for assessing genetic diversity among crop genotypes. Recently, high resolution melt analysis (HRM) has been reported for detecting genetic variability. However, there is limited information on the use of HRM in conjunction with other molecular marker techniques for assessing genetic variation in sorghum [*Sorghum bicolor* (L.) Moench]. This study was conducted to compare random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers with HRM analyses to determine genetic variation among selected sorghum genotypes. Eight diverse sorghum accessions obtained from the plant genetic resources, Department of Agriculture, Forestry and Fisheries/South Africa were subjected to both analyses. DNA was extracted from fresh leaves of the eight accessions and amplified using three RAPD and three SSR primers. The HRM analysis was performed and temperature normalised melting curves and difference plots were created and results compared. Both the molecular markers and HRM revealed variations among the accessions. The HRM melting profiles fairly well correlated with results from the RAPD and SSR analysis. The clustering of sorghum accessions using SSR marker highly corresponded with the HRM analysis. Therefore, the HRM can be a useful tool in genetic diversity and classification of sorghum genotypes without post-PCR analysis or processing.

Key words: Genetic diversity, high resolution melt analysis, RAPD, simple sequence repeat, sorghum.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important food security crops worldwide after wheat, rice, maize, and barley (Poehlman, 1994). It is grown by small and large scale farmers for food and livestock feed including environments considered to be marginal for other cereal crops such as maize and wheat. In spite of its economic potential, there has been limited research and extension in sorghum compared to other cereal crops such as maize and wheat in South Africa (Wenzel et al., 2001).

Characterisation of sorghum germplasm is an important aspect in plant breeding programs to find new sources of genetic variation. Exploitation of diversity at the genotypic

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level requires an efficient system such as molecular marker technology (Iqbal et al., 2010). This technology allows estimation of genetic resource diversity more efficiently and reliably than phenotypic markers which are subject to genotype by environment interaction (Staub et al., 1997). The use of molecular markers aid the conventional breeding in many aspects including selection of parents for hybridization through genotypic diversity analysis studies (Jain and Kharkwal, 2004).

The DNA-based fingerprinting techniques are important tools for genetic variation studies in plant breeding and germplasm management and gene identification (McGregor et al., 2000; Simionic et al., 2002). Various marker techniques have been used for analysis of genetic diversity in sorghum including random amplified polymorphic DNA (RAPD) (Prakash et al., 2008; Iqbal et al., 2010; Shivjee Sah and Khanna, 2010) and simple sequence repeat (SSR) (Shehaz et al., 2009; Rajput et al., 2012; Reddy et al., 2012). These marker techniques are discrete, co-dominant or dominant, and free from epistatic gene action (Tanksley et al., 1989; McIntyre et al., 2001). In contrast to morphological or biochemical marker techniques, DNA-based methods are independent of environmental factors result in a high level of polymorphism (Karp et al., 1997).

The high resolution melt (HRM) analysis is helpful for discriminating genotypes in combination with other molecular marker techniques. This technique has been reported in various studies and is advantageous due to lack of post-polymerase chain reaction (PCR) sample processing and/or separation (Montgomery et al., 2007). The HRM can be used for genotyping in various ways including the use of amplicon melt with temperature controls (Seipp et al., 2007), unlabelled probes with the 3'-end to prevent extension by *Taq* polymerase (Zhou et al., 2004), and fluorescently labeled primers. The most common approach is the use of fluorescently labeled primers whereby the primers bind the genomic DNA in the presence of a dye.

The fluorescence is captured while samples are melting, following the PCR. The melting profiles are determined by GC content, length and the homozygosity and/or heterozygosity status of the sample genotypes (Reed et al., 2007). The melting profiles can be evaluated by normalized fluorescence curves, derivative plots, or difference plots to detect variation (Vaugn and Elenitoba-Johnson, 2004). This takes a very short time which is more beneficial to marker assisted selection in plant breeding programmes.

The HRM has been reported in various studies including maize (Naidoo, 2010), potato (De Koeyer et al., 2010), almond (Wu et al., 2009), apple (Chagne et al., 2008), white lupin (Croxford et al., 2008), and perennial rye grass (Studer et al., 2009). The objective of the study was to compare RAPD and SSR marker and high resolution melt (HRM) analyses to determine genetic variation among selected sorghum genotypes.

MATERIALS AND METHODS

Plant materials

Eight sorghum genotypes were used for this study supplied by the plant genetic resources unit of the Department of Agriculture, Forestry and Fisheries (South Africa). Lines were coded as KZ87, EC67, NW93, LP48, FS52, EC3217, FS489 and MP65 and originally collected from KwaZulu-Natal, Eastern Cape, North West, Limpopo, Free State, and Mpumalanga provinces as prefixed in the codes. The sorghum genotypes were planted in the African Centre for Crop Improvement (ACCI) tunnel at the University of KwaZulu-Natal. Two sorghum seeds of each accession were planted in 40 cm diameter plastic pots filled with seedling mix in five replicates. Each line was planted in five pots. Cultural practices were applied as necessary and water supplied as drip irrigation.

Deoxyribonucleic acid (DNA) extraction and quantification

The DNA was extracted using the CTAB extraction method (Kang et al., 1998; Saghai-Maroof et al., 1984). Young fresh leaves of each sorghum genotype were sampled via a lid of 1.5 ml microfuge tubes where a disc of leaf material was punched out into a tube. 400 µl of 2% CTAB extraction buffer [2% (w/v) CTAB 100 mM of Tris-HCl (pH 8.0), 1.4 M of NaCl, 20 mM of EDTA (pH 8.0) and 1% (w/v) PVP] was added to each tube. Samples were treated with a bead beater. The leaf material was then incubated in a water bath at 66°C for 1 h. 400 µl of chloroform: isoamyl alcohol (24:1 v/v) was added and then centrifuged at 4°C for 10 min at 12 000 *g.* The top aqueous part was transferred into fresh 1.5 ml microfuge tubes and 0.62 volumes of ice-cold isopropanol was added, mixed gently and incubated at room temperature to precipitate the DNA. The precipitated product was centrifuged for 5 min at 12 000 *g* and the supernatant was removed. The DNA pellet was washed by adding 1 ml 70% (v/v) ethanol followed by centrifugation at 12000 *g*. The supernatant was discarded and following a quick spin the pellet was aspirated and then air dried at room temperature overnight. The DNA was suspended in 25 µl TE buffer. The genomic DNA concentrations were quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA) with absorbance 260/280 nm wavelengths. The DNA was diluted to a working concentration of 25 ng/µl TE buffer.

PCR and HRM conditions

The stock solution of the template DNA for each sample was diluted to a final concentration of 25 ng/µl in 0.1× TE buffer. A total of 15 µl per reaction mixture was prepared for PCR amplification. The reaction mixture contained 4.5 µl water, 7.5 µl KAPA (2×) Universal (KAPA SYBR$^{(R)}$ FAST qPCR Kit) master mix, 1.5 µl of 500 nM RAPD primers (Table 1) and the 1.5 *µ*l of 25 ng/*µ*l template DNA. Negative and positive controls were included in each PCR setup to ensure non-contamination of the reagents. The reaction mixtures were amplified using a Rotor-Gene 6000 real-time rotary analyser. The machine was also used for HRM analysis. The PCR was performed as follows: an initial step of 95°C 2 min followed by 40 cycles of 95°C 10 s, 37°C 20 s, and 72°C for 20 s. The HRM was performed after the PCR analysis with the ramp temperature of 70 to 90°C with each step rising with 0.2°C; the pre-melt conditioning was 90 s for the initial step, and 2 s equilibration after each step. The melting curves were created and the Rotor-Gene software was used to distinguish sorghum accessions by normalisation and difference plots.

A total of 15 µl reaction mixture of each accession was prepared as described for the previous method but using the forwards and reverse SSR primers at 200 nM (Table 1). The PCR setup was

Table 1. List of SSR and RAPD primers used for the experiment.

Marker	Primer		Sequence
SSR	GWKZN43	Xtxp335	Forward: 5´ TATTTCCTCTTGAAAGAATCAGGG 3´
	GWKZN44	Xtxp335	Reverse: 5´ TATTCATCGAGCAAAAGGCA 3´
	GWKZN45	Xtxp258	Forward: 5´ CACCAAGTGTCGCGAACTGAA 3´
	GWKZN46	Xtxp258	Reverse: 5´ GCTTAGTGTGAGCGCTGACCAG 3´
	GWKZN47	Xtxp145	Reverse: 5´ GTTCCTCCTGCCATTACT 3´
	GWKZN48	Xtxp145	Reverse: 5´ CTTCCGCACATCCAC 3´
RAPD		OPA-12	5´ TCGGCGATAG 3´
		OPA-16	5´ AGCCAGCGAA 3´
		OPA-18	5´ AGGTCACCGT 3´

95°C for 2 min for the initial denaturation temperature, followed by 95°C for 10 s, 72°C for 20 s for 40 cycles. The HRM was performed at ramp temperature 75 to 85° with each step rising with 0.1°, and the pre-melt conditioning for 90 s on the initial step, and 2 s interval after each step. The melting curves were normalised and the Rotor-Gene software was used to distinguish sorghum accessions by difference plots.

Gel electrophoresis, data collection and analysis

About 2 µl of the amplified PCR products, 3 µl of water and 1 µl of loading buffer were mixed and loaded onto an agarose gel. A low molecular weight DNA ladder (6x) [1× Gel loading dye, blue: 2.5% Ficoll-400, 11 mM EDTA, 3.3 mM Tris-HCL (pH 8.0 at 25°C), 0.017% SDS, and 0.015% bromophenol blue] was also included in the reaction. For RAPD analysis, the samples were electrophoresed on a two and half percent (w/v) agarose gel in a 1× TBE buffer for 1 h 40 min with the current of 150 voltage. For the SSR analysis, the samples were electrophoresed on a 2% (w/v) agarose gel in a 1× TBE buffer for 1 and half hours with the current of 150 V. Bands were visualized by staining the gel in an ethidium bromide 0.5 µg/ml for 25 min and destained in water. The detained gel was then photographed under UV light. Bands were scored as either present (1) or absent (0). Dendrograms were constructed using a dice coefficient analysis (Dice, 1984) in NTSYS v2.1 software (Numerical Taxonomy and Multivariate Analysis) Computer Programme.

RESULTS

RAPD analysis

Agarose gel analysis of RAPD marker

Polymorphisms were observed in all primer sets used for RAPD marker analysis. The fragment sizes ranged from 200 bp to more than 700 bp for the RAPDs (Figure 1). A high molecular weight DNA ladder could have been used for estimating the fragment sizes. Different banding patterns were observed among the genotypes. OPA 12 did not amplify very well and the bands associated with OPA 18 were difficult to score although some levels of polymorphism were observed, hence were not considered. The OPA 18 primer seemed to amplify the same region in all genotypes except for the MP65 accession. However, problems with data scoring and the reproducibility in amplification of RAPD markers have been reported (Jones et al., 1998).

The numbers of bands scored are shown in Table 2. When comparing the RAPD and the SSR, the RAPD marker system revealed more polymorphic bands than the SSR. The number of polymorphic bands for RAPD was up to 12 whereas for SSR marker it was 5.

High resolution melt analysis for RAPD marker

The melting profiles were created and normalised for three RAPD primers (Figure 2). The temperature normalised melting curves displayed the change in the fluorescence to 100% with the temperature of 76°C and 0% fluorescence at about 86°C. Variation was observed in all the three RAPD primers. OPA12 showed differences among the sorghum genotypes studied.

Genotypes EC67, FS489 and EC3217 had similar melting curves. The NW93 had a different curve compared to other genotypes followed by LP48. This could be due to early maturity, and short stature of the accession itself. Genotypes FS52 and EC3217 had similar normalised melting profiles. Also, NW93 and LP48 had similar normalised melting profiles. The genotypes that had similar melting profiles are closely related. These genotypes with unique profiles from others can be selected and used in breeding programmes as sources of valuable genes. Although some variations were observed among the melting curves, the curves created were difficult to interpret for OPA 16 and OPA 18 RAPD primers.

Similar results were observed in the different plots. Genotype MP65 was used as a baseline with a threshold confidence level set at 90% in a difference plot analysis. The results are shown in Table 2. Genotypes EC67, and FS489 were very closely related with the baseline entry, MP65. NW93 was not related to MP65 and the rest of the genotypes. LP48 distantly related with MP65 and other

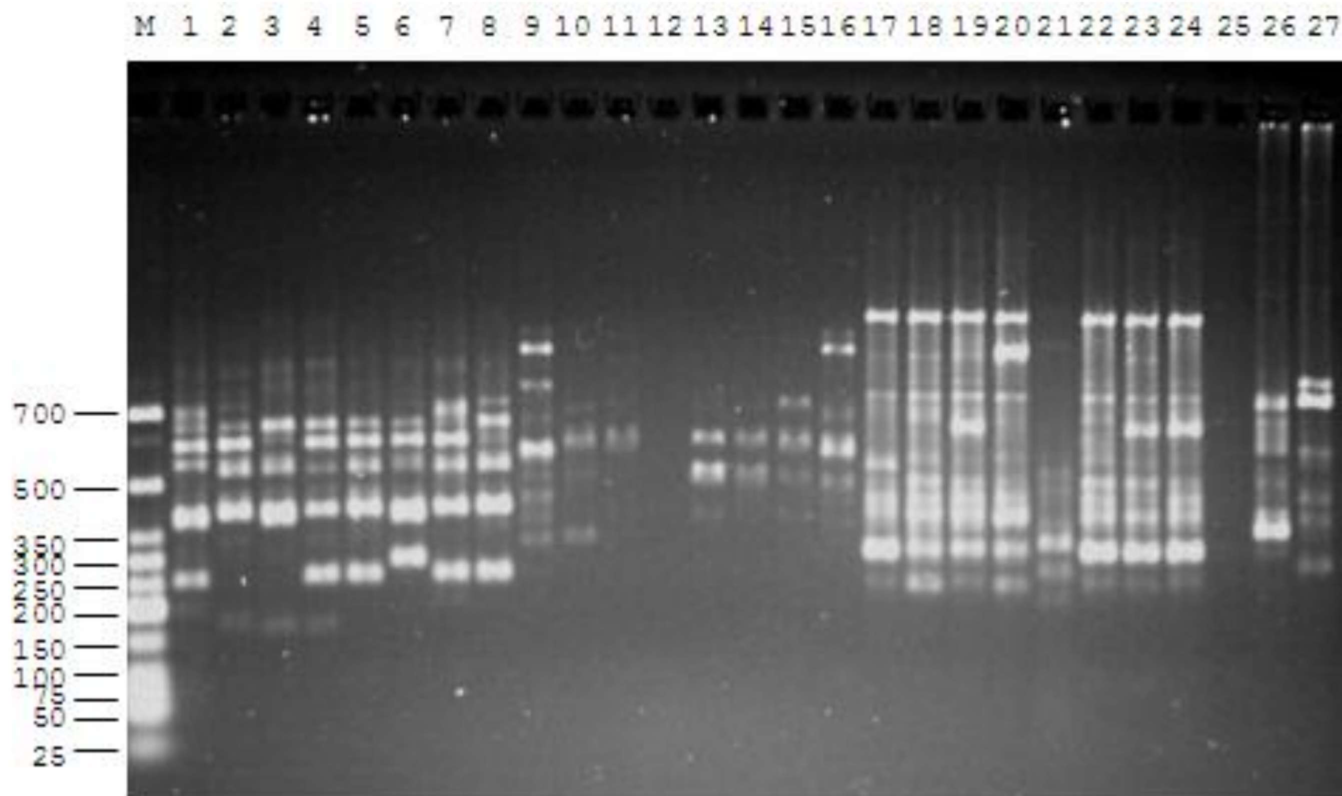

Figure 1. Agarose gel electrophoregrams of three RAPD primers (OPA-12, OPA-16, and OPA-18 from left to right). M = molecular weight ladder; 1= KZ87; 2= FS52; 3=NW93; 4= EC67; 5=MP65; 6= LP48; 7= FS489; 8= EC3217; and the same lines were replicated in that order from 9-16 and 17-24 across the remaining two markers; number 25 represents the no temperate control; 26-27 represent the control (human DNA).

Table 2. Similarity of sorghum genotypes from difference plot of HRM analysis.

Genotype	KZ87	FS52	NW93	EC67	MP65	LP48	FS489	EC3217
Confidence (%)	66.56	80.41	19.98	93.07	100	41.31	97.09	77.01

accessions. Although different from the baseline and other genotypes, FS52, EC3217 and KZ87 appeared to be closely related.

Analysis using the SSR

Gel electrophoresis

Polymorphisms were observed among sorghum genotypes studied using three SSR primer pairs. The sizes of the SSR fragments ranged from 150 to 300 bp (Figure 3). The Xtxp 248 primer showed monomorphic banding pattern with only EC67 showing a small difference in fragment size.

Polymorphic bands were scored for both SSR and RAPD marker analyses. The numbers of bands scored are shown in Table 3. The RAPD marker system revealed more polymorphic bands than the SSR. The number of polymorphic bands for RAPD was up to 12 whereas for SSR marker analysis it was 5.

High resolution Melt analysis for SSR marker

The normalised temperature melting profiles were created in the HRM analysis. Differences were observed among normalised melting profiles in all three SSR primer pairs. In this study, only data on one SSR primer, Xtxp 335 was interpreted. The Xtxp 335 primer showed clear variation among the genotypes tested (Figure 4). KZ87 showed a different melting curve from the entire set of sorghum accessions. Genotypes FS52, FS489 and EC3217 had very similar melting pattern. Similar melting profiles were also observed for genotypes EC67 with MP65, and NW93 with LP48. These genotypes melting

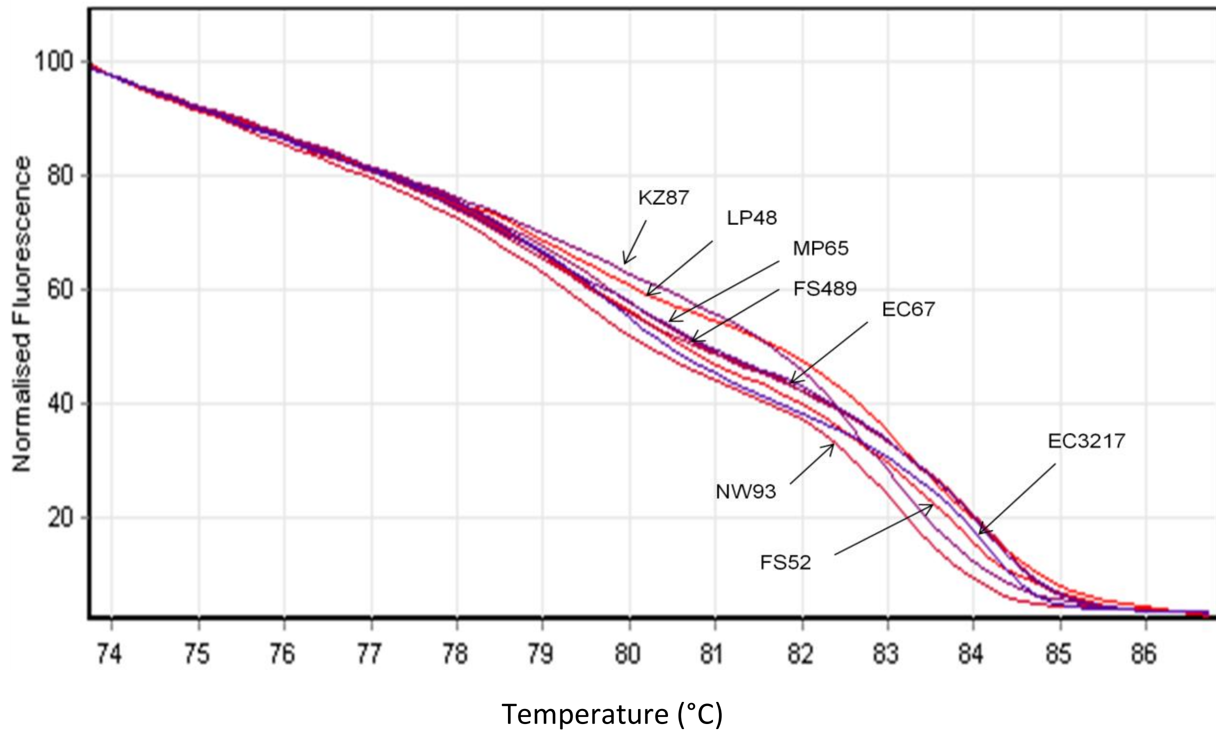

Figure 2. The temperature normalised HRM melting curves of sorghum genotypes using RAPD OPA 12 primer.

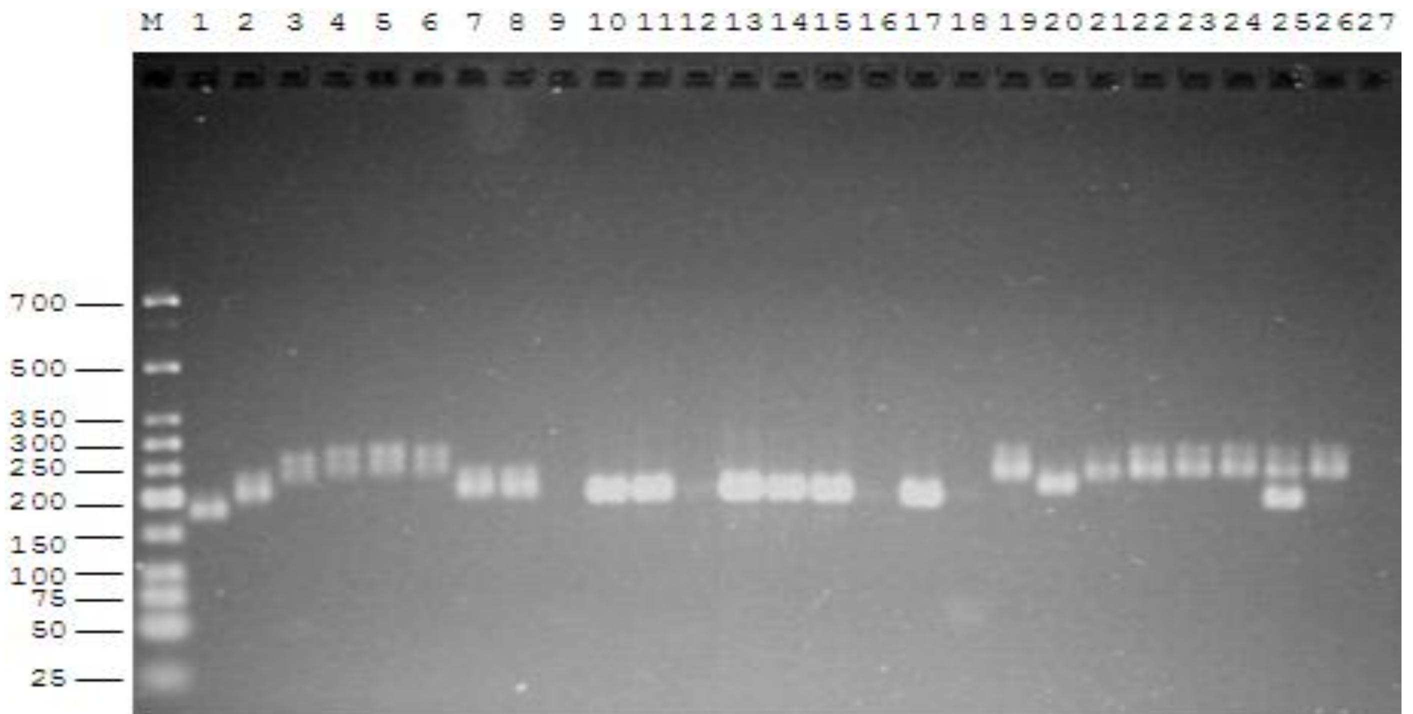

Figure 3. Agarose gel electrophoregrams of three SSR primers (Xtxp335, Xtxp145, and Xtxp258- from left to right). M = molecular weight ladder; 1= KZ87; 2= FS52; 3= NW93; 4= EC67; 5= MP65; 6= LP48; 7= FS489; 8= EC3217; and the same lines were replicated in that order from 11-18 and 20-27 across the remaining two markers; numbers 9, 18, and 27 represent the no template control.

Table 3. Number of bands scored for five primers showing polymorphism.

Marker/primer		Number of bands scored for each sample							
		KZ87	FS52	NW93	EC67	MP65	LP48	FS489	EC3217
SSR	Xtxp335	1	1	2	2	2	2	1	1
	Xtxp258	1	1	0	1	1	1	0	1
RAPD	*OPA12*	6	4	4	5	5	5	5	6
	OPA16	3	1	1	0	2	2	3	4
	OPA18	4	5	5	4	1	4	5	4

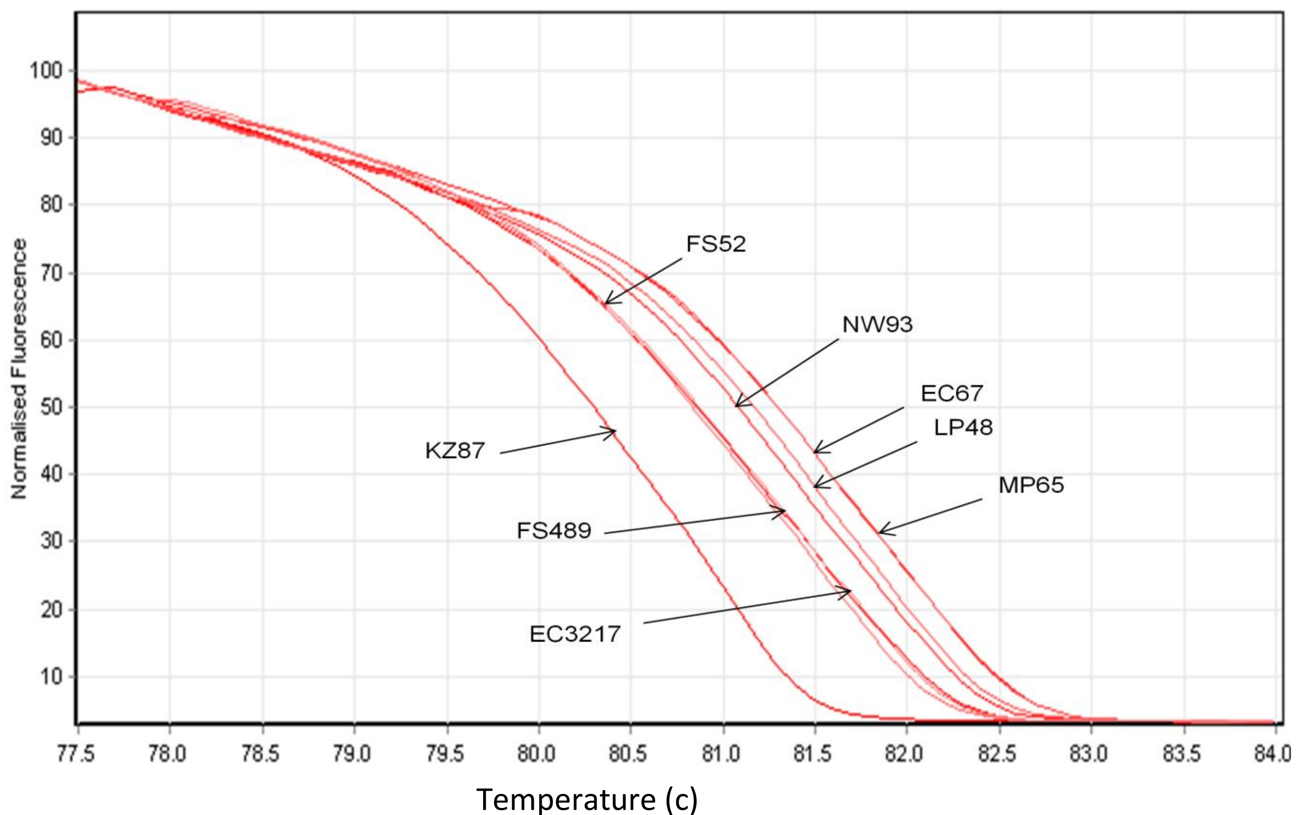

Figure 4. The temperature normalised HRM curve analysis of sorghum genotypes using SSR primer, Xtxp335.

Table 4. Similarity of sorghum genotypes from difference plot of HRM analysis.

Genotype	KZ87	FS52	NW93	EC67	MP65	LP48	FS489	EC3217
Confidence (%)	0.21	94.79	33.24	2.03	1.9	14.62	100	94.64

profiles were different from the EC3217, FS52 and FS489.

The genotype FS489 was used as a reference genotype in the difference plot analysis with the threshold confidence level set at 90%. The results are presented in Table 4 and are similar to the normalised melting curves (Figure 4). Accessions FS52, and EC3217 were closely related to the reference genotype, FS489. KZ87 followed by MP65, EC67, LP48 and NW93 accessions were distantly related to all other sorghum accessions. These genotypes can be grouped together and FS52 and EC3217 can also fall into the same group with the baseline genotype, FS489.

Melting profiles were also created for the two SSR

primer pairs, Xtxp 258 and Xtxp 145. Differences were observed among the sorghum genotypes when Xtxp 258 and Xtxp 145 primer sets were used although most seemed to have similar melting profiles (Figure 4). This suggests that either the marker had been monomorphic for that particular locus and hence incapable of discriminating the melt peaks or may not be possible to see the polymorphism at the given resolution.

Clustering of eight accessions by RAPD and SSR analysis

RAPD

The RAPD markers grouped the sorghum genotypes into two clusters (Figure 5). The first cluster was divided into two groups where KZ87, FS489, and EC3217 were similar with genetic similarity value of 1.00, and EC67 and MP65 were also 100% similar. The second cluster grouped FS52, NW953 and LP48 together. FS52 and NW93 were the same and LP48 varied. LP48 could be a useful candidate as a parent in breeding programmes. The RAPD analysis showed a matrix with the genetic distances from 0.72 to 1.00 among the genotypes under study (Table 5).

SSR

Variation was observed among sorghum genotypes when SSR markers were used. Dice similarity matrix was used to cluster accessions using the UPGMA algorithm. The resulting dendrogram revealed two clusters (Figure 6). The first cluster grouped FS489 together with FS52 and EC3217 which were closely related. The second cluster was divided into two sub-clusters; where LP48, MP65 and EC67 were the same with a genetic distance of 1.0 and were similar to NW93. Although clustered together, KZ87 was distantly related to NW96, EC67, MP65 and LP48. The SSR analysis showed genetic similarity values ranging from 0.33 to 1.00 among the genotypes tested (Table 6).

DISCUSSION

Variation was observed among the genotypes studied when using SSR and RAPD analyses. In the SSR analysis, monomorphic banding pattern observed, implied that the primer amplified the same region in the genome. Further, an alternative gel such as acrylamide could be useful due to its good resolution power compared to agarose. The heterozygotes were observed among the genotypes which concurred with the ability of this technique to distinguish among homozygosity and heterozygosity status of the genotypes.

In RAPD analysis, a large number of bands were revealed due to the random priming nature and potential confounding effects associated with co-migration with other markers (Tessier et al., 1999). Several studies compared various molecular marker systems in sorghum and the SSR was highly correlated with the morphological markers in contrast to AFLP (Geleta and Labuschagne, 2005). Panwar et al. (2010) compared the efficiency and effectiveness of RAPD and the SSR markers in finger millet. Similar studies for comparison of marker systems were also reported by Agrahama and Tuinstra (2003).

The HRM analysis showed existence of genetic variation among the sorghum genotypes when SSR and RAPD primers were used. The melting profiles and difference plots grouped genotypes for SSR and RAPD similarly. The genotypes denoted in various groups can be selected for crosses as potential parents to undertake selection. Similar studies were performed in sweet cherry where HRM was used together with SSR to distinguish sweet cherry cultivars (Ganopoulos et al., 2011).

Olive cultivar genotyping was performed using HRM and SSR (Muleo et al., 2009). Mackay et al. (2008) reported microsatellite high resolution melt analysis as useful tool for variety identification and verification in olive and grapevine plants. The use of HRM in conjunction with RAPD has been reported in parasitological studies thus far (Tulsiani et al. 2010). However, in other studies, the use of the HRM analysis was reported effective and efficient when used with other molecular markers (De Koeyer et al., 2010; Naidoo, 2010). For instance, Hofinger et al. (2009) reported the accuracy and sensitivity of the HRM analysis for predicting wide range of nucleotide polymorphisms in barley.

In the RAPD analysis, the genetic distances ranged from 0.72 to 1.00. The results concur with Grenier et al. (2000) who reported diversity range of 0.71 to 0.93. The RAPD marker analysis showed some distinction among the genotypes and most of them were very similar.

Ayana et al. (2000) also reported weak differentiation of Ethiopian and Eritrean sorghum accessions. The results of the RAPD clustering appear to correspond fairly with the melting profiles of the HRM analysis (Figure 2). On the other hand, the SSR analysis showed genetic similarity values ranging from 0.33 to 1.00. When SSR and RAPD analysis were compared, similarity matrices constructed based on shared allele analysis revealed the lowest average genetic similarity between genotypes when estimated using SSR markers (0.33) and was higher among entries when determined using RAPD markers (0.72). These results indicate that RAPD markers provide less resolving power than SSR markers.

The accessions that are distantly related can be selected and used for crossings as parents for use in a breeding programme, estimation of genetic advance as well as further improvement of sorghum cultivars. The results appear to show a wide diversity among the

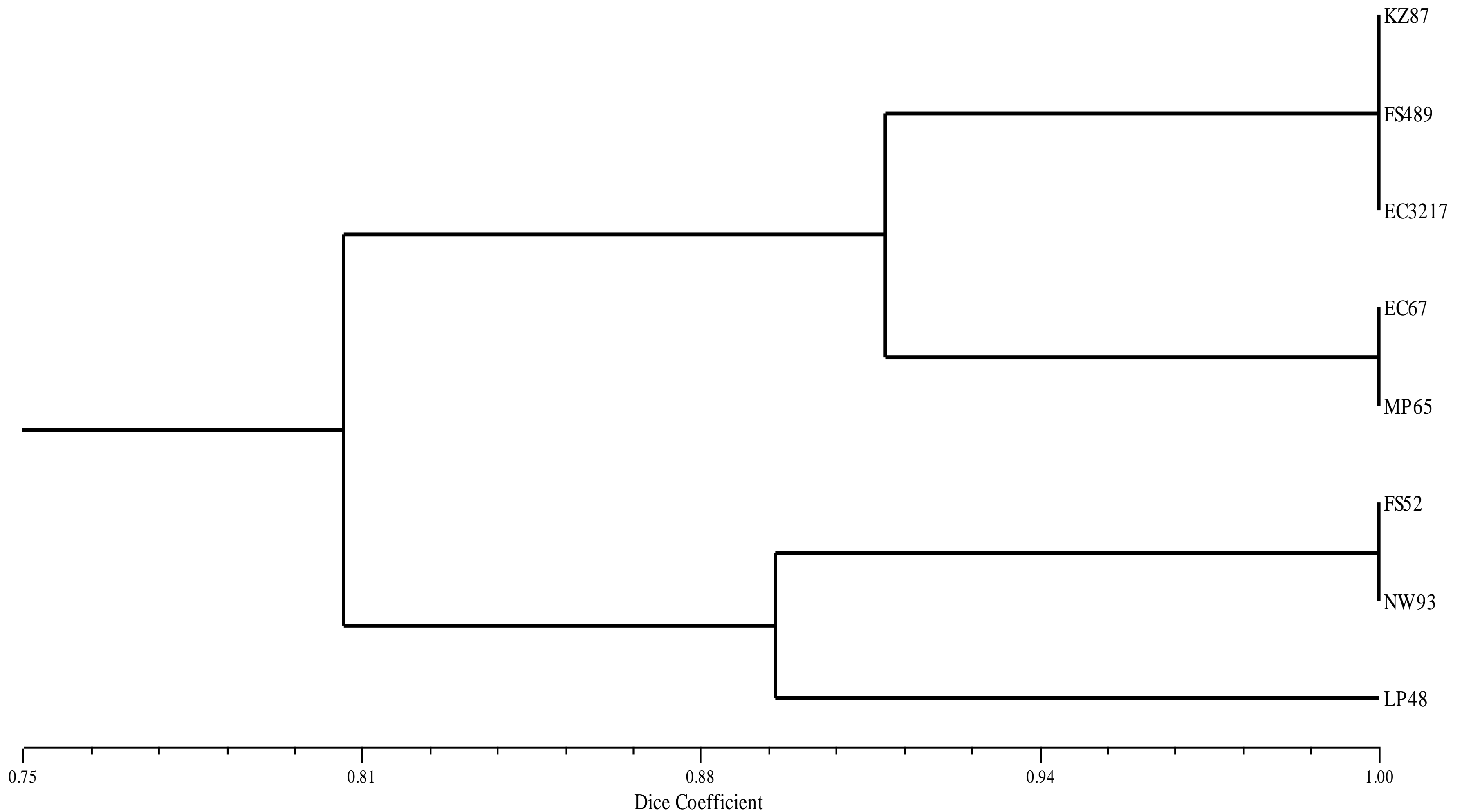

Figure 5. Dendrogram constructed based on RAPD marker showing genetic distance and cluster groups among eight sorghum accessions.

sorghum genotypes. Dje et al. (2000) estimated a wide genetic diversity when analysing 25 sorghum landraces derived from a restricted area of North Western Morocco with three SSR markers.

Conclusion

The SSR and RAPD markers as well as HRM analysis revealed genetic variation among the sorghum genotypes. The HRM was useful in detecting variation among the sorghum genotypes using melting profiles and corresponded well with the SSR clustering of sorghum genotypes. Hence,

Table 5. Dice similarity coefficient for SSR analysis on eight sorghum accessions.

Genotype	KZ87	FS52	NW93	EC67	MP65	LP48	FS489	EC3217
KZ87	1.0000							
FS52	0.8000	1.0000						
NW93	0.8000	1.0000	1.0000					
EC67	0.9091	0.8889	0.8889	1.0000				
MP65	0.9091	0.8889	0.8889	1.0000	1.0000			
LP48	0.7273	0.8889	0.8889	0.8000	0.8000	1.0000		
FS489	1.0000	0.8000	0.8000	0.9091	0.9091	0.7273	1.0000	
EC3217	1.0000	0.8000	0.8000	0.9091	0.9091	0.7273	1.0000	1.0000

Table 6. Dice similarity coefficient for SSR analysis on eight sorghum accessions.

Genotype	KZ87	FS52	NW93	EC67	MP65	LP48	FS489	EC3217
KZ87	1.0000							
FS52	0.3333	1.0000						
NW93	0.4000	0.0000	1.0000					
EC67	0.6667	0.3333	0.8000	1.0000				
MP65	0.6667	0.3333	0.8000	1.0000	1.0000			
LP48	0.6667	0.3333	0.8000	1.0000	1.0000	1.0000		
FS489	0.3333	0.3333	0.4000	0.3333	0.3333	0.3333	1.0000	
EC3217	0.6667	0.6667	0.4000	0.6667	0.6667	0.6667	0.6667	1.0000

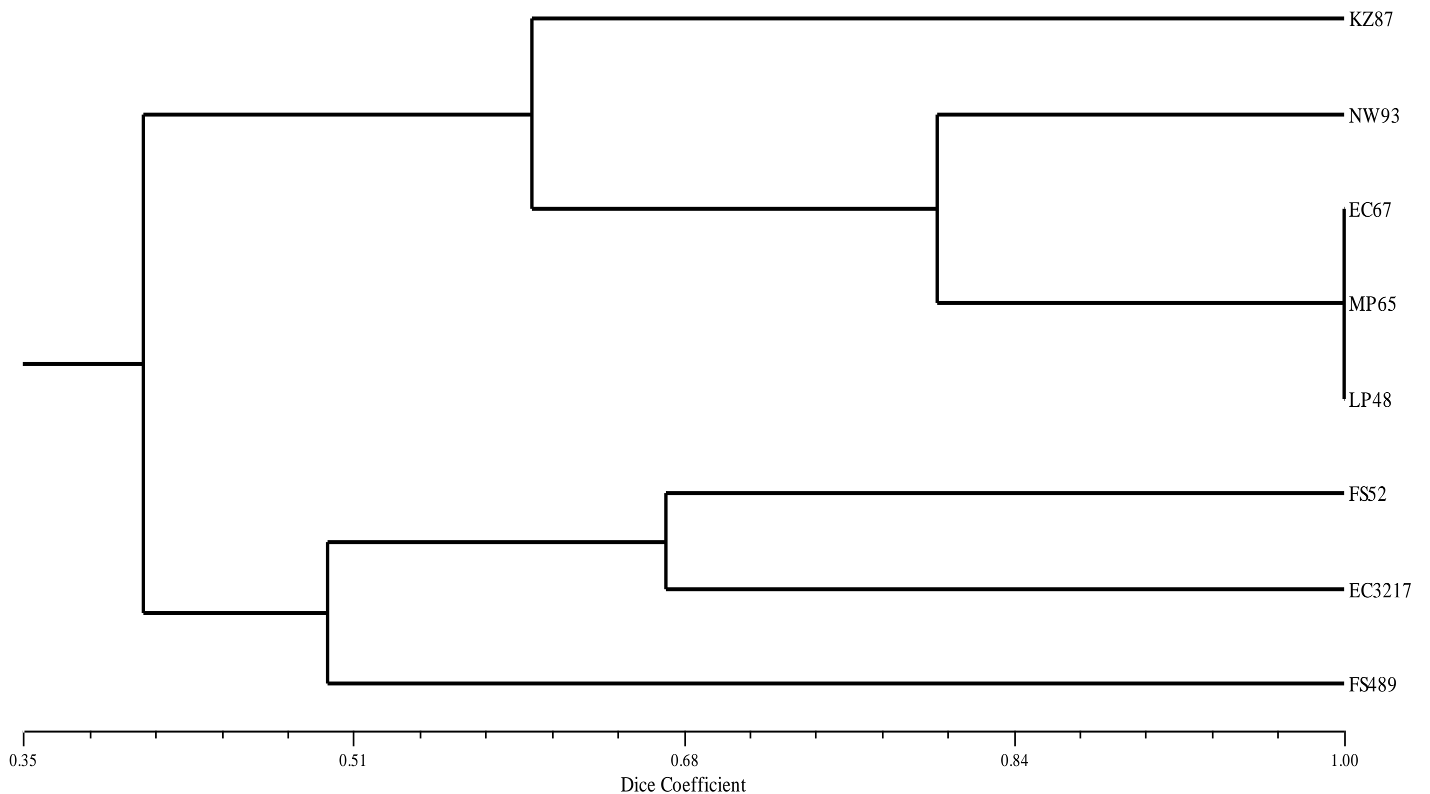

Figure 6. Dendrogram constructed based on SSR data, showing genetic distance and cluster groups among eight sorghum accessions.

the HRM can be useful for genetic diversity analysis and assigning sorghum genotypes into heterotic groupings without post-PCR analysis or processing. It can be useful in accelerating selections in plant breeding programmes as is timely, efficient and effective and can be extended with other molecular marker techniques.

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