Full Length Research Paper

Real time PCR mediated determination of the spontaneous occurrence of *Sorghum bicolor* alleles in wild sorghum populations

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The study evaluates the utility of Real Time PCR (RT-PCR) in quantitative and qualitative analysis of alleles in sorghum populations and the spontaneous occurrence of *Sorghum bicolor* alleles in wild populations of sorghum. Leaf and seed material from wild sorghum accesions were sampled in Homabay, Siaya and Busia counties to represent Western Kenya sorghum producing regions. A second sampling was done on S_2 populations of *S. bicolor*, *Sorghum halepense* and *Sorghum sudanense* maintained in the greenhouse. Crop loci were evaluated in all materials using a LightCycler® 2.0 system. Real Time PCR was effective in qualitative and quantitative determination of crop alleles in both crop and weedy backgrounds of *S. sudanense*, *S. halepense* and *S. verticilliflorum.* Crossing point values ranged between 19.7 from 30 ng template to 35.9 from 0.015 pg of template on locus SB1764. Melting peaks analysis ranged between 83.29 to 88°C on locus SB1764 and between 86.01 to 80.88°C on locus SB3420 effectively differentiating the 4 species. RealTime-PCR was successful in quantitative and qualitative analysis of specific crop alleles from loci SB1764 and SB3420 from seed and leaf DNA. Spontaneous occurrence of crop and rare alleles in wild sorghum populations growing in sympatry with crop cultivars showed the presence of crop and rare alleles in wild sorghum populations. Means of wild populations from lower midland$_1$ (LM_1), LM_2, LM_3 and LM_4 AEZs were not significantly different. It is therefore vital to test *S. bicolor* seeds and other plant materials in transit, at entry points and populations of growing plants for foreign genes including transgenes using RT-PCR.

Key words: Real time polymerase chain reaction (*PCR*), *Sorghum bicolor*, *Sorghum halepense*, *Sorghum sudanense*.

INTRODUCTION

Populations of crop and weedy sorghums have been shown to coexist in sympatry in most sorghum producing regions (Doggett, 1988). The members of this genus have shown great potential for gene flow through interspecific hybridization. This situation has lead to the existence of crop alleles in wild sorghum, attributed to ancient and recent hybridizations (Morrell et al., 2005; Mutegi et al., 2009). These crop alleles in weedy populations have

contributed to the wide variability maintained in populations of sorghum through disruptive selection process (Doggett and Majisu, 1968). The existence of domesticated sorghum alleles in wild sorghums can easily be followed by the use of PCR applications. However, conventional PCR has several technical limitations that hinder its maximum potential. The deficiencies in traditional PCR are associated with the speed, sensitivity, reliability and inability to adequately quantify target DNA (Gachon et al., 2004; Arya et al., 2005). This has led to the development of more quantitative and precise methods of analyzing target DNA sequences such as RT-PCR. The method uses instrumentation and fluorescent dyes that permit online monitoring of PCR amplification in real time (Heid et al., 1996; Wittwer et al., 1997).

Real Time-PCR offers rapidity, sensitivity, and specificity due to its ability to amplify targeted sequences from small amounts of starting DNA (Gachon et al., 2004; Bustin, 2000; Arya et al., 2005). The technique has been improved relative to conventional PCR with regard to its ability to quantify with precise calculation the starting amount of template and estimate DNA quantity during amplification (Hernandez et al., 2003; Gachon et al., 2004). In addition, RT-PCR has the capacity to differentiate the amplified strands, by applying the melting temperature (TM) approach. This is an important application in the genotyping of genetic material.

In plant studies RT-PCR has the potential of identifying genetic variability and the subsequent introgression of genes into a cultivar of interest (Gachon et al., 2004). Real Time-PCR has been applied in the absolute and relative qualitative determination of the presence of foreign DNA in plant material (seeds, leaves, roots) (Gachon et al., 2004). Foreign DNA routinely assayed through RT-PCR in plant populations include; pathogens such as bacteria, fungi and viruses (Deepak et al., 2007; Sankaran et al., 2010). The technique has the potential of detecting polyploidy events, subsequent chromosomal inversions and induced or spontaneous mutations in populations. Recently, transgenic events have also been detected through RT-PCR (Gachon et al., 2004; Deepak et al., 2007).

Transgene copy number has previously been assayed in transgenic material using duplex real time PCR (Ingham et al., 2001). German et al. (2003) showed the efficiency of the technique in differentiating hemizygous and homozygous transgenic plants with single copy inserts. In addition, MON 810 in transgenic maize was evaluated using a specific RT-PCR detection system that was shown to be a specific and accurate Taqman based technique (Hernandez et al., 2003; Pla et al., 2006). Qualitative and quantitative determination of transgene events has been reported in canola (Yang et al., 2006). Simplex and duplex RT-PCR was successfully applied in quantification of GMO events in maize and soybean (Alary et al., 2002). Multiplex RT-PCR has also been developed for simultaneous detection of *Cry/A(b)* and *pat* genes of transgenic maize insect resistant events namely, 176, MON810, BT11 and T25 of transgenic maize (Permingeat et al., 2002). In addition RT-PCR technique has been useful in quantification of the L1781 ACCASE inhibitor resistance allele in DNA from leaf and seed pools of weedy Lolium populations (Kaundun et al., 2006). The technique can serve as a quick substitute to Southern blots for analysis of copy numbers of alleles or loci under investigation. RT-PCR can therefore be useful in tracking down unintended introgression of crop alleles into weedy backgrounds. This is necessary in the Eastern Africa region due to the presense of several wild and weedy sorghum species in the crop sorghum production zones. The current study evaluated spontaneous hybridization in conspecific sorghum species using RT-PCR.

MATERIALS AND METHODS

Experimental materials were obtained from two sources; first, leaf samples were obtained from S_2 populations of *S. bicolor*, *S. halepense* and *S. sudanense* grown in the greenhouse. Secondly, wild sorghums in three counties of Kenya namely Homabay, Siaya and Busia representing Western Kenya sorghum producing regions around Lake Victoria were sampled. Sampling units within the counties were clustered based on the mapped agro-ecological zones (AEZs). Sorghum growing farms were randomly sampled and a total of 175 samples were collected representing AEZs in the three counties (Table 1). The samples were collected after seed set in farms that had both weedy and crop sorghums. Weedy sorghums growing on road reserves and uncultivated land in the clusters were also sampled. The seeds and first two leaves next to the panicle were collected labelled and kept on ice and transported to the laboratory located at the College of Agriculture and Veterinary Sciences (CAVS) (-1° 14' 59.72", +36° 44' 30.79") of the University of Nairobi.

Sample preparation and DNA extraction

Young leaves were collected from 2 to 4 week old plants in the greenhouse, while old leaves were obtained from green sections of physiologically mature plants in the field. Thirty mature seeds were randomly sampled per panicle, dried and bulked. Genomic DNA from leaf and seed samples was extracted through two methods. First, genomic DNA was extracted from young leaves by using a modified CTAB extraction procedure (Doyle and Doyle, 1990; Barnaud et al., 2008). Total nucleic acid was extracted from the seeds, by a modified CTAB based protocol (Delobel et al., 2007). Agarose gel electrophoresis for genomic DNA extracted from young leaf, old leaf and seed tissues was done before RT-PCR to establish the quantity and quality of the DNA. The RT-PCR products were separated and validated using a 4% UltraPure™ Agarose gel from Invitrogen ™. Densitometric analysis of agarose gels was used to determine the original concentration of genomic DNA.

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Table 1. Agro-ecological zone characteristics in Homabay, Siaya and Busia counties of Kenya where wild –weedy sorghums were collected.

Agro-ecological Zone	Altitude (m)	Ann. Mean Temp (^{o}C)	Ann. Mean Rainfall (mm)	Number of samples
Busia (+0° 28' 45.07", +34° 7' 11.39")				
Lower Midlands (LM)				
LM1	1200-1440	22.2-21.0	1650-2000	18
LM2	1200-1350	22.3-21.4	1420-1800	13
LM3	1140-1500	22.7-21.0	1100-1450	16
LM4	1135-1200	22.7-22.3	900-1100	14
Homabay (-0° 36' 15.25", +34° 29' 57.86")				
Upper Midlands (UM)				
UM1	1500-1570	21.0-20.5	1600-1900	11
Lower Midlands (LM)				
LM1	1300-1500	21.8-20.9	1500-1900	9
LM2	1200-1350	22.3-21.5	1400-1600	8
LM3	1140-1250	22.7-22.0	1020-1390	12
LM4	1135-1200	22.7-22.3	890-1020	21
LM5	1135-1180	22.7-22.4	-	
Siaya (+0° 2' 18.43", +34° 12' 19.88")				
Lower Midlands (LM)				
LM1	1300-1550	21.7-20.4	1600-1800	11
LM2	1300-1550	21.7-20.4	1300-1600	12
LM3	1140-1550	22.7-20.4	900-1500	11
LM4	1140-1450	22.7-20.9	800-1300	19
LM5	1135-1300	22.7-21.7	700-900	

Farm management hand book of Kenya (2005, 2009).

Table 2. Primer sequences developed for Real Time PCR analysis from the *Sorghum bicolor* physical map that exhibit polymorphism on the number and sizes of alleles present

Marker name	Chromosome	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
SB3258	5	ATTGTTGTCCTCCCCTCCACC	AGTACCTGAACCAGGCGTCGCT
SB1764	3	CTTGTGCTTGCTTGCACCATATTC	GTCGATGAGGAGCTTCATGCTCAG
SB3008	5	ATTTTGGTCCAGTTGCTCTTGCAT	CGTACGTGAGCGAGGATTGTTCTA
SB3420	6	GAGCCAGCATGCATGATAATTGTT	CACAAAGGCATGACAGTCAATCAA
SB5058	9	GAGAATTGGAAGAAAGCCTCGGTT	CAGAGCTCCTAAACGGTCCTCAAA
SB5458	10	AATGTGGTGGTGTTGTGTCTCCAT	TCTACTGCTATCATCGCCTCCACC
SBI000X	10	CTAGAGGATTGCTGGAAGCG	CTGCTCTGCTTGTCGTTGAG

Primers were designed to give a 300 bp fragment in *S. bicolor,* with a melting temperature of between 60 and 62 degrees centigrade.

Primer design

Primers were designed based on the polymorphic SSR sequences from crop sorghum physical map genomic clones on five linkage groups chromosomes found in Phytozome databases (http://www.phytozome.net/sorghum). To ensure the specificity of the primers prior to synthesis, e-PCR was performed for all primer combinations. Annealing sites were also confirmed by using BLASTn procedure against the NCBI GenBank database. The primers selected for the study are shown in Table 2. The polymorphism in the primers were designed on presence of bands in *S. bicolor* and absence of the same in the other members of the sorghum genus.

Real time pcr assay

The RT-PCR was performed on a LightCycler® 2.0 carousel based system in 20 μL final volume. Crop specific primer pairs for loci SB1764, SB3008, SB3420, SB5058 and SBI000X were used in the assay. The PCR reaction was optimized to obtain high end point fluorescence and low cycle threshold or crossing point (Cp) values
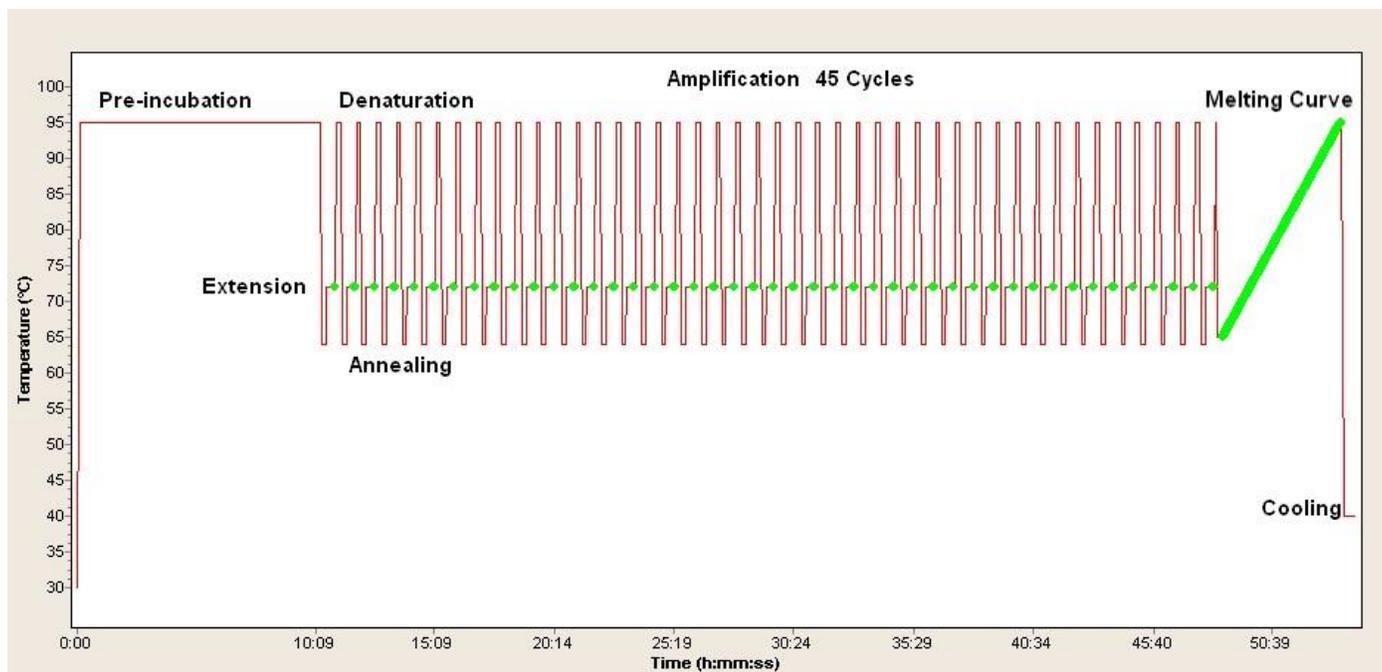
Figure 1. Reaction conditions interface on the LightCycler® 2.0 thermocycler detailing the temperatures and time for each of the stages and steps.

by varying the concentrations of the target primers, template concentrations and reaction conditions. PCR reactions were done in 20 µL of final reaction consisting of 1.5 mM $MgCl_2$, 1 µM of primer mix, 1X master mix in LightCycler® 2.0 capillaries. Reaction mixtures were prepared from a LightCycler® Faststart DNA master SYBR green 1 Kit containing 62 µL of reaction mix. Vial 1a was thawed away from light and 10 µL of the enzyme from vial 1b was added. This was mixed gently by pipetting up and down to avoid degrading the enzyme. The Master Mix was shielded from light with aluminium foil and stored in a pre-cooled LightCycler® centrifuge adapters cooling block. A PCR mix for ten samples was prepared with 169.6 µL molecular grade water, 4.4 µL 25 mM $MgCl_2$, 1 µL each of primer 1 and primer 1 and 22 µL of the Master Mix.

LightCycler® capillaries were placed in the numbered adapters and 18 µL of the PCR mix pipetted into each of the ten capillaries. 2 µL of the DNA sample was added to the PCR mix and centrifuged at 700 × g for 5 s. The capillaries were then placed in the numbered slots on the carousel for the reaction. The LightCycler® was programmed to amplify the target DNA for qualitative analysis, quantitative analysis and melting curve analysis through three stages. A pre-incubation stage was set at 95°C for 10 min. The second stage involved quantification through three steps; denaturation was done at 95°C for 10 s followed by annealing at 64°C for 10 s and extension at 72°C for 20 s. A single reading was acquired after this stage through each of the 45 cycles. The third stage was set for a continuous reading at the melting stage 95°C after annealing for 15 s at 65°C. The temperature transition rate/slope/ ramp rate was maintained at 20°C per sec except during melting when it was maintained at 0.1°C per sec. After melting, the reaction was cooled to 40°C for 30 s (Figure 1).

Data analysis

Fluorescence detected at 530nm was monitored at annealing and data were analyzed using the LightCycler® Software 4.x. Crossing

points (CP) were compared and utilized to evaluate the original template concentration. The presence or absence of amplification and the determination of DNA concentration approaches were useful in explaining the presence of crop alleles in wild sorghums. Fluorescence was measured at 530 nm from a SYBR Green background giving a standard amplification curve in the exponential phase, linear and plateau phase. The original template concentration was quantified during the geometric phase by analysis of the threshold cycles or crossing points (CP) cycles values. Melting curve analysis was vital for the determination of the similarity or differences of the RT-PCR products as well as the origin of alleles in the population. Melting peak analysis was also applied in genotyping the populations of wild sorghum from the three counties from Western Kenya. End point analysis was done to estimate the final amount of amplified DNA by the fluorescence emitted and the density of the band on PCR gel electrophoresis. Discriminant analysis was done in GENSTAT 14 (VSN, 2012) to evaluate the differences of populations from different AEZs in the sampled counties. The Mahalanobis (D-Squared) (Mahalanobis, 1930; GENSTAT 14, VSN 2012) test was performed to evaluate differences between subpopulations (groups) based on the crossing point and melting peak values for the wild sorghums from the sampled counties.

RESULTS

The utility of real time PCR in tracking alleles in sorghum populations

Real time PCR amplified fragments from sorghum accessions of crop and weedy backgrounds for poly-morphic *S. bicolor* loci (Figure 2). Qualitative detection was done for locus SB3420 in the nine samples, where all samples showed the presence of the allele except in
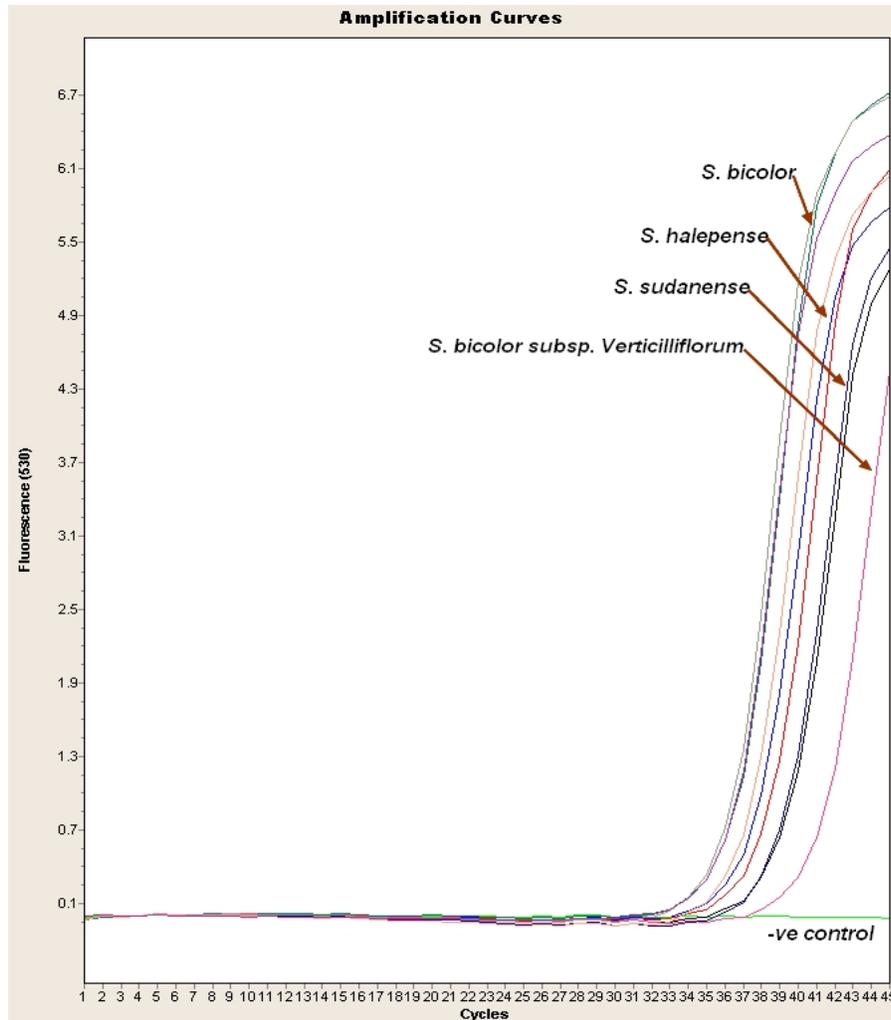
Figure 2. Real Time PCR amplification of locus SB3420 in nine sorghum samples and one negative control containing water.

the case where water replaced the sample to form the negative control (Figure 2). Using loci SB3420, amplification curves were obtained in RT-PCR experiments from templates that were known to possess the primer annealing sequences. In the reaction, *S. bicolor* samples had CP values ranging from 28 to 29 cycles, *S. halepense* had 31 to 32 cycles, *S. sudanense* had 33 to 34 cycles and *S. verticilliflorum* had 34 cycles. Samples from *S. bicolor* had higher template concentration than *S. halepense* and *S. sudanense* (Figure 2) and therefore the emitted fluorescence exceeded the standardized threshold before other species showing had higher CP values.

Quantitative detection of crop loci SB1764 in *S. bicolor* template dilutions

Sensitivity of the RT-PCR was evaluated on *S. bicolor*

templates of different concentrations (Figures 3 a, b). Serially diluted templates in T. E buffer showed differences on the CP values. Locus SB1764 positioned on chromosome 3 was amplified in all *S. bicolor* templates except in the negative control that had water. Differences in CP values among template dilutions (DNA:T.E. buffer) of 1:4, 1:5, 1:6 and 1:7 were less than those exhibited among serial dilutions of x100, $x10^{-1}$, $x10^{-2}$, $x10^{-3}$, $x10^{-4}$ and $x10^{-5}$ (Figure 3a, 3b, Table 3). In the reactions the 1:4 template dilutions showed CP values ranging between 19.7 and 22.58 cycles; 1:5 *S. bicolor* template dilutions had CP values of 23.10 to 24.89 cycles. 1:6 template dilutions had CP values of 24.4 to 25.53 cycles. 1:20 template dilution had CP value of 27.56 cycles. Crossing point values varied with DNA concentrations in serial template dilutions. A ten times (x10) dilution corresponding to 15 ng determined through densitometry showed a CP value of 26.11 cycles; $x10^{-1}$ dilution corresponding to 1.5 ng showed a CP value of 30.55
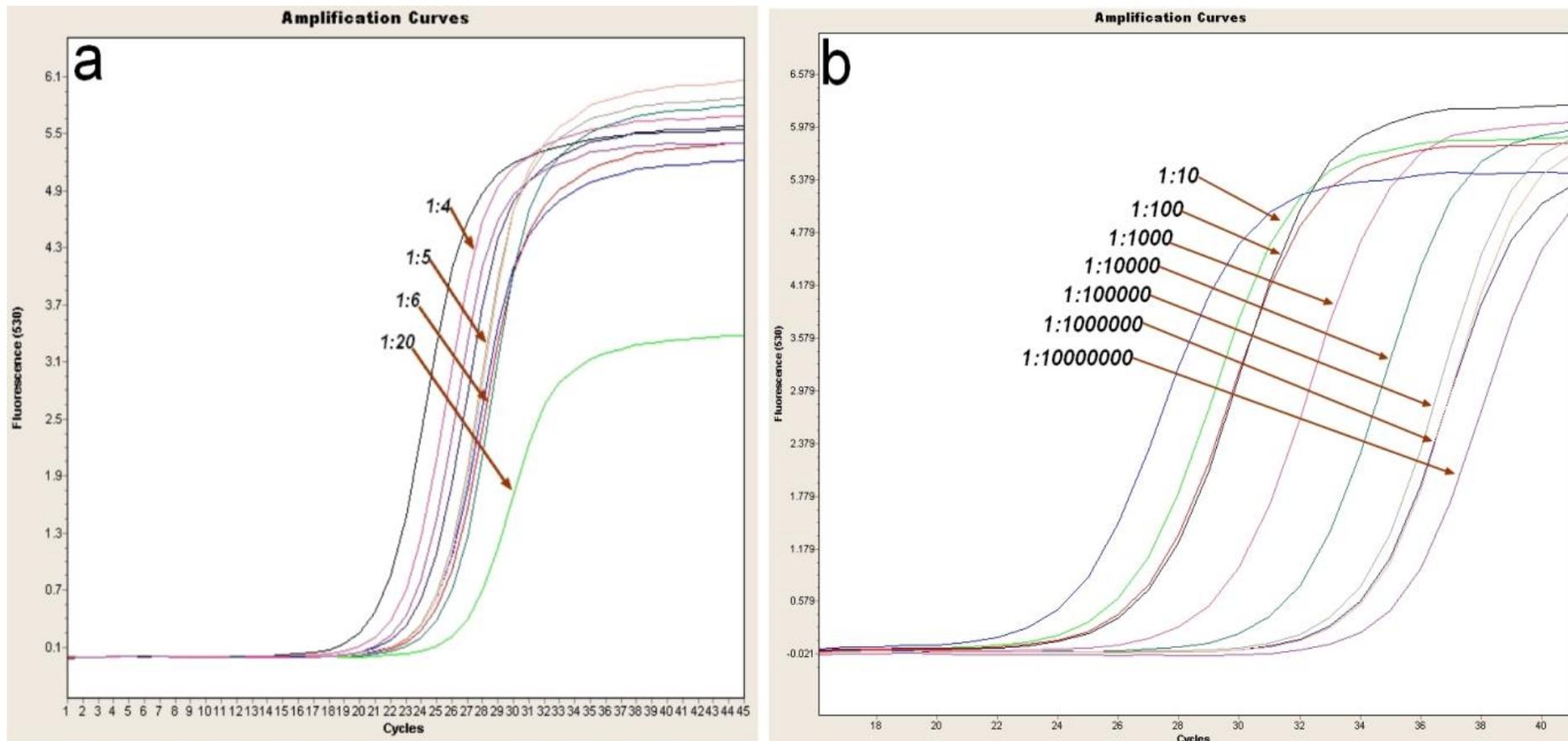
Figure 3. (a) Sensitivity of the Real Time PCR in amplifying crop specific loci in S. bicolor. Templates used were three samples of *S. bicolor* diluted at 1:4, 1:5, 1:6, 1:20- in TE. Primers targeting locus SB1764 were used. **(b)** Templates used were serial diluted (x100, x10-1, x10-2, x10-3, x10-4 and x10-5) in TE. Primers targeting locus SB1764 were used.

cycles; template dilution of $x10^{-2}$ (0.15 ng) had a CP value of 32.70 cycles; while that of $x10^{-3}$ (0.015 ng) gave a CP value of 32.53 cycles (Figure 3b, Table 3). Further dilution of the template showed a higher CP value, this was seen when template dilution of $x10^{-4}$ (1.5 pg) gave a CP value of 32.87 cycles (Figure 3b and Table

3). A dilution of $x10^{-5}$ corresponding to 0.15 pg gave a CP value of 33.84 cycles while a dilution of $x10^{-6}$ corresponding to 0.015 pg of DNA had a CP value of 35.90 cycles (Figure 3b and Table 3). *S. bicolor* template concentration was regressed to the CP values to develop a standard curve; Y= -0.4295X + 32.95 with an R^2 of 0.929.

End point analysis was performed to determine the fragment sizes amplified on 4% agarose gels. A 300 bp band was recovered from *S. bicolor* sample dilutions ranging from 30 ng to 0.015 pg, except the negative control (Figure 4) Denser bands were seen on agarose gel electrophoresis from samples that had higher starting template

Table 3. Precision of Real Time PCR in detection of sorghum template dilutions

Dilution from ~150ng template	Locus SB1764
	Cp values (Cycles)
1:4 (37.5 ng)	19.7-22.58
1:5 (30 ng)	23.10-24.89
1:6 (25 ng)	24.4-25.53
1:20 (7.5 ng)	27.56
1:10 (15 ng)	26.11
1:100 (1.5 ng)	30.55
1:1000 (0.15 ng)	32.70
1:10000 (0.015 ng)	32.53
1:100000 (1.5 pg)	32.87
1:1000000 (0.15 pg)	33.84
1:10000000 (0. 015 pg)	35.90
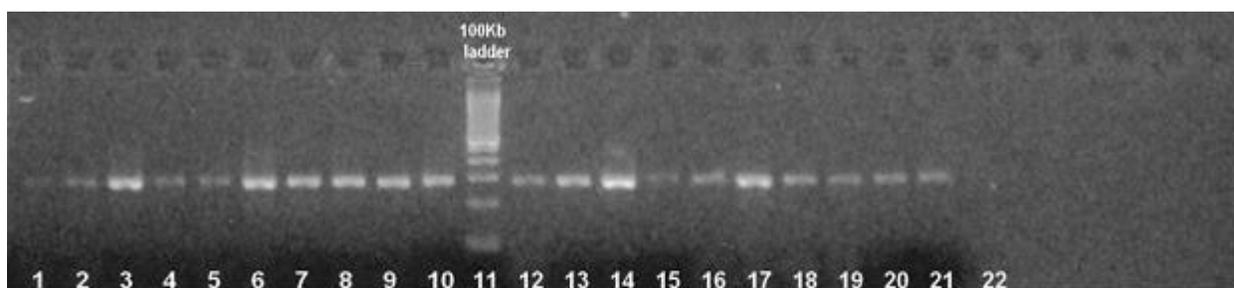
Figure 4. End point validation of amplification, of locus SB1764, showing the expected 300bp fragment from *S. bicolor* accessions using the Real time PCR - Light cycler® 2.0 (Roche). The bands corresponding to concentrated templates (lanes 3, 6, 7, 8, 9, 13, 14 and 17) are denser. Water replaced DNA in lane 22.

Table 4. Sensitivity of Real Time PCR in detection and characterization of sorghum accessions (*S. bicolor*, *S. halepense*, *S. sudanense* and *S. bicolor ssp. verticilliflorum*).

Loci species	SB1764		SB3420		SB3258	
	CP	TM (°C)	CP	TM (°C)	CP	TM (°C)
S. bicolor	32.96	88.00	30.63	86.01	21.45	81.43
S. sudanense	35.96	84.09	35.42	80.96	20.25	82.24
S. halepense	31.21	84.65	31.96	80.49	22.88	81.56
S. verticilliflorum	32.51	83.92	30.84	80.88	-	-

SB, S. bicolor; SS, S. sudanense; SV, S. verticilliflorum; SH, S. halepense; CP, crossing point/threshold cycle; TM, melting temperature (□C).

concentrations (above 1.5 ng) than those that were serially diluted. This validated the PCR amplification and confirmed the effect of template concentration to the product of PCR amplification (Figure 4).
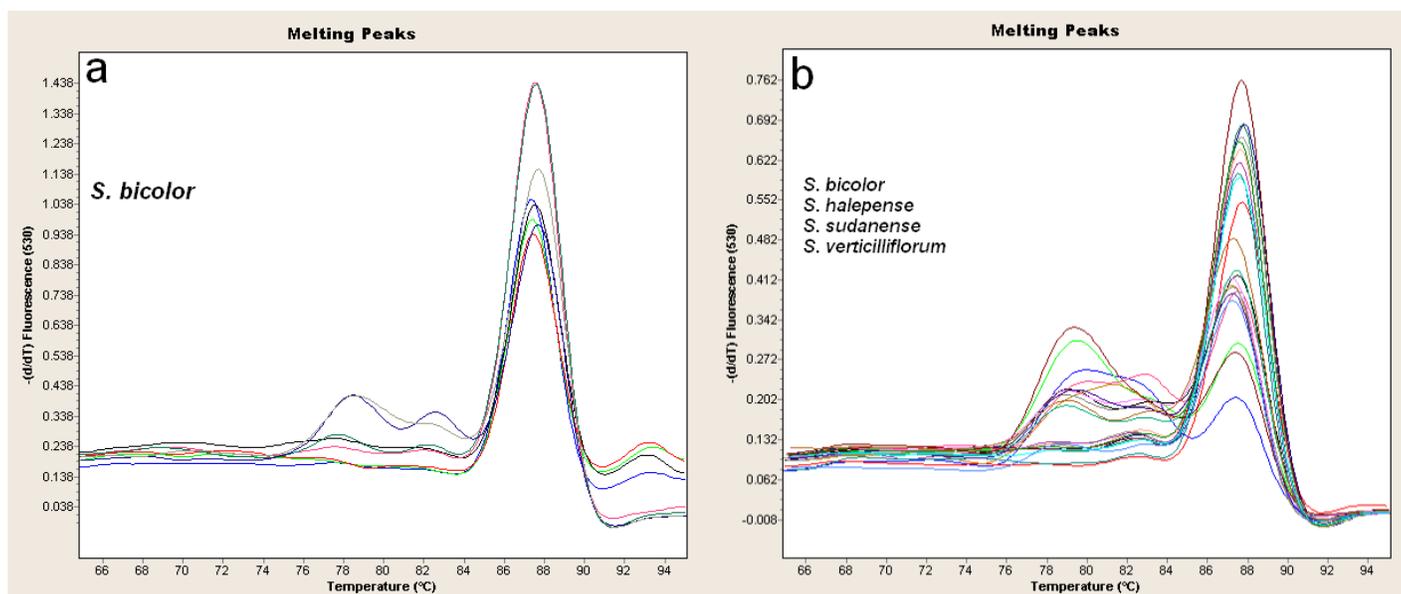
Amplification and melting curve analysis for *Sorghum bicolor, Sorghum halepense, Sorghum sudanense* and *Sorghum bicolor* ssp. *verticilliflorum*

S. bicolor, *S. halepense*, *S. sudanense* and *S. verticilliflorum*

samples were evaluated using the RT-PCR technique (Table 4). Three loci SB1764, SB3420 and SB3258 were targeted in the templates and CP values from qualitative evaluation were compared. Analysis of locus SB1764 in *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* gave CP of 32.96, 35.96, 31.21 and 32.51 cycles respectively (Table 4). Almost similar CP values were obtained with locus SB3420 in *S. bicolor* (30.63 cycles), *S. sudanense* (35.42 cycles), *S. halepense* (31.96 cycles) and *S. verticilliflorum* (30.84 cycles). Amplification of the SB3258 locus had lower CP
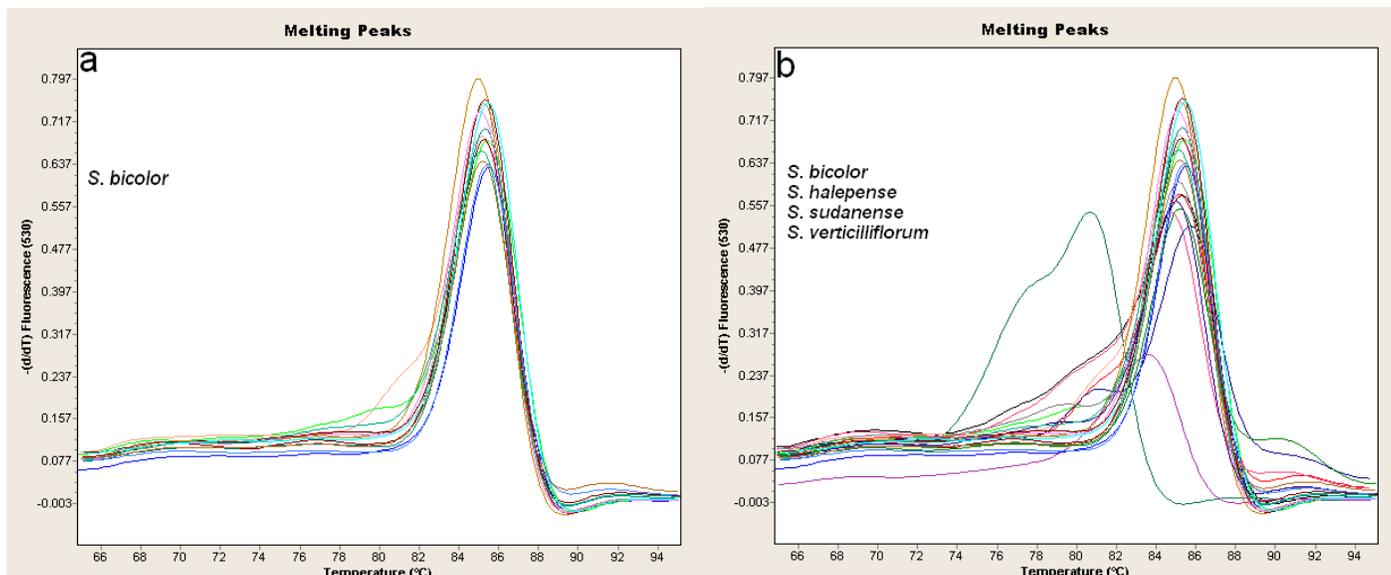
Figure 5. Melting curve analysis for *S. bicolor* templates amplified with primers targeting locus SB3420. A peak was seen at 86°C (a). For weedy sorghum accession templates, variation in melting peaks from 76 to 88°C were seen (b).

Figure 6. Melting curve analysis for *S. bicolor* templates amplified with primers targeting locus SB1764, showing peak at 88°C (a). For weedy sorghum accession templates, variation in melting peaks from 76 to 88°C were seen (b).

values of 21.45 cycles in *S. bicolor*, 20.25 cycles in *S. sudanense* and 22.88 cycles in *S. halepense* (Table 4, Figure 5). Absolute differences in CP values were attributed to the starting concentration of the template since varying yields were possible for DNA extracted from different samples.

The melting curve analysis showed differences in the three loci of the sorghum acessions (Figure 5, Figure 6

and Table 4). *S. bicolor* had a peak at 88°C for locus SB1764 (Figure 6), 86.01°C for locus SB3420 (Figure 5) and 81.43°C for locus SB3258 (Table 4). *S. sudanense* showed a peak at 84.09°C for locus SB1764, 80.96°C for locus SB3420 and 82.24°C for locus SB3258. *S. halepense* had a peak at 84.65°C for SB1764, 80.49°C for SB3420 and 81.56°C for SB3258. *S. verticilliflorum* had a peak at 83.92°C for locus SB1764 and 80.88°C for

Table 5. Real Time PCR in qualitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, seed, old leaf).

Tissue	SB3420					SB1764				
	Species									
	SB		SS	SV	SH	SB		SS	SV	SH
	CP	TM (°C)	CP	CP	CP	CP	TM (°C)	CP	CP	CP
Young leaf	23.46	85.34	25.74	24.65	21.29	20.51	87.91	20.70	20.81	20.88
Seed	23.62	85.40	24.84	21.73	25.96	20.88	87.57	20.93	20.85	20.84
Old leaf	23.12	85.32	22.13	20.60	23.76	20.81	87.58	20.86	20.91	20.84

SB, *S. bicolor*; SS, *S. sudanense;* SV, *S. verticilliflorum;* SH, *S. halepense;* CP, crossing point/threshold cycle; TM, melting temperature (°C).
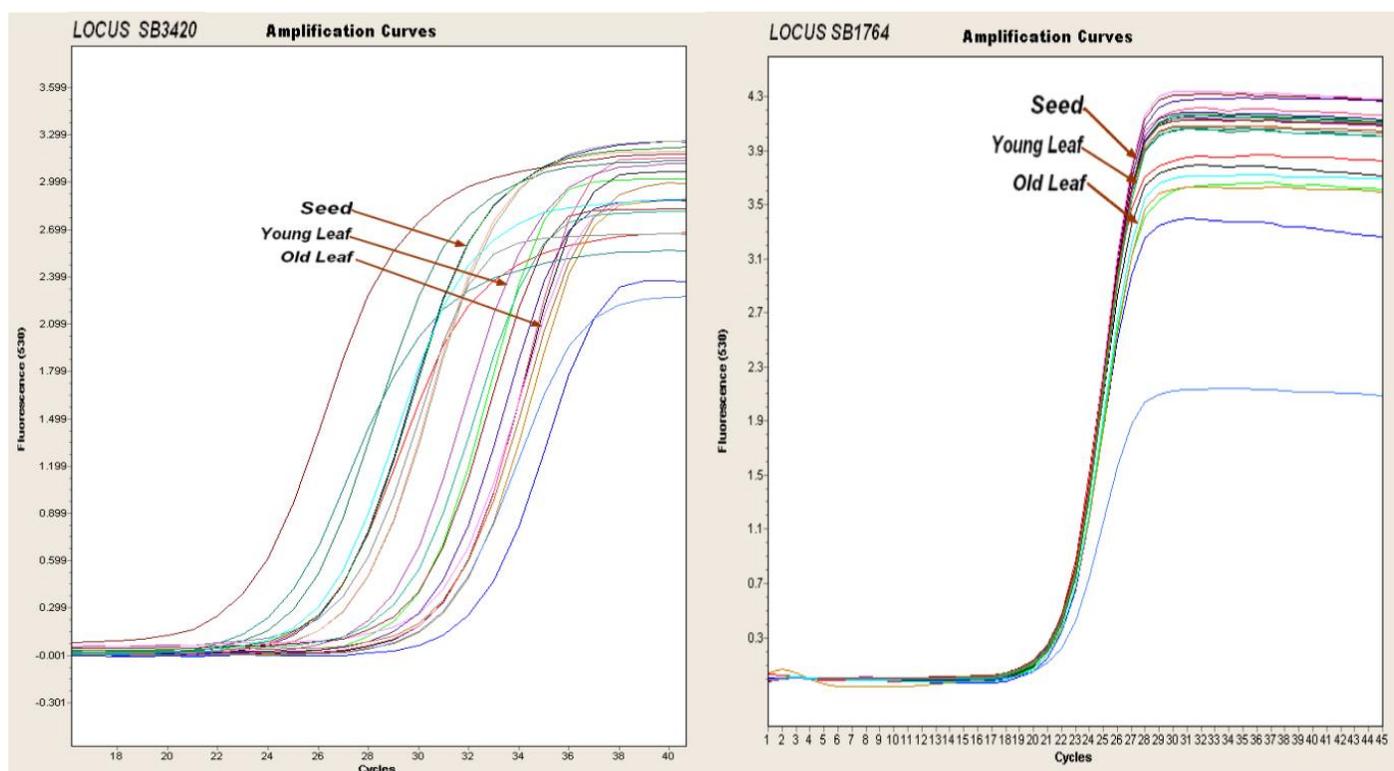
Figure 7. Real Time PCR amplification curves of crop specific loci (SB3420 and SB1764) from different plant tissues (leaf, seed and old leaf) of twenty sorghum samples.
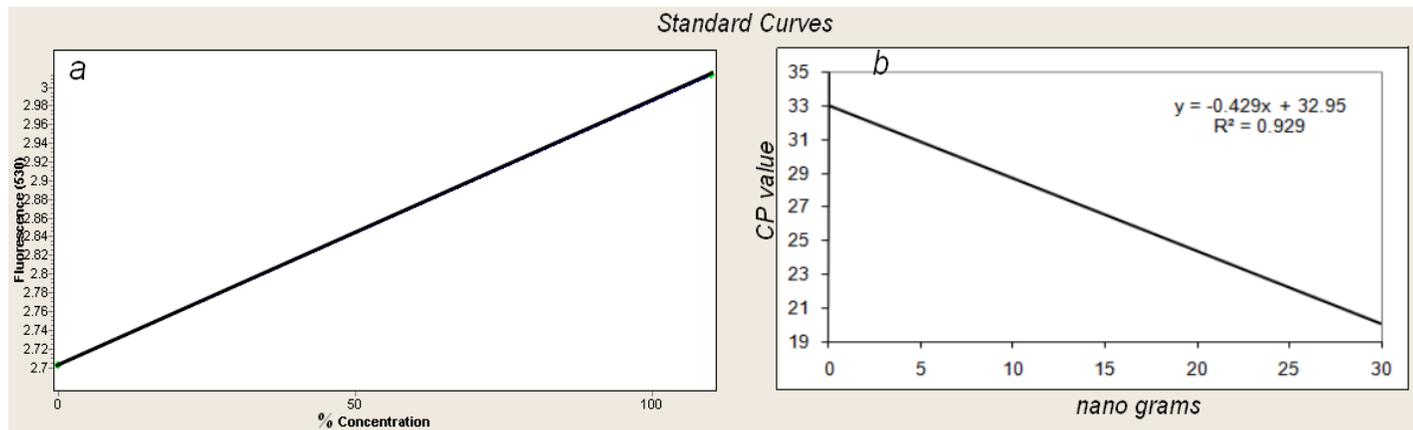
locus SB3420 (Figure 5, 6 and Table 4).

Analysis of real time PCR efficiency in the qualitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, old leaf and seed) of sorghum species

Using RT-PCR, templates from young leaf, old leaf and dry seed were successfully amplified from loci SB1764 and SB3420. Qualitative determination of *S. sudanense*, *S. halepense* and *S. verticilliflorum* tissues yield the expected CP and TM values (Table 8).

Using SB1764 on *S. bicolor* accessions, CP values of 20.51, 20.88 and 20.81 were obtained from young leaf derived template; seed derived template and old leaf derived template respectively (Table 5). The melting temperature peaks were in the expected range from *S. bicolor* of 87.91°C, 87.57°C and 87.58°C from young leaf, seed and old leaf respectively. In *S. sudanense*, CP values of 20.70 cycles, 20.93 cycles and 20.86 cycles were obtained from young leaf seed and old leaf respectively on locus SB1764 (Figure 7).

Amplification of locus SB1764 from *S. verticilliflorum* yield CP values of 20.81 cycles, 20.85 cycles and 20.91 cycles respectively from young leaves, seed and

Figure 8. Standard curves showing increase in fluorescence (530 nm) with increase in concentration (a); and increase in CP values (b) with decrease in template concentration in nanograms

Table 6. Real Time PCR in quantitative determination of crop alleles (SB3420 and SB1764) from different plant tissues (young leaf, seed, old leaf).

Tissue	SB3420								SB1764							
	Species															
	SB		SS		SV		SH		SB		SS		SV		SH	
	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng	CP	ng
Young leaf	23.5	22.1	25.7	16.8	24.7	19.3	21.3	27.1	20.5	29.0	20.7	28.5	20.8	28.3	20.9	28.1
Seed	23.6	21.7	24.8	18.9	21.7	26.1	26.0	16.3	20.9	28.1	20.9	28.0	20.9	28.2	20.8	28.2
Old leaf	23.1	22.9	22.1	25.2	20.6	28.8	23.8	21.4	20.8	28.3	20.9	28.1	20.9	28.0	20.8	28.2

SB, *S. bicolor*; SS, *S. sudanense;* SV, *S. verticilliflorum;* SH, *S. halepense;* CP, crossing point/ threshold cycle; ng, nano grams.

old leaves (Figure 7 and Table 5). The expected melting temperatures were obtained confirming species identity. In *S. halepense*, locus SB1764 was amplified from young leaf tissues, seed and old leaf tissues to yield CP values of 20.88 cycles, 20.84 cycles and 20.84 cycles respectively (Figure 7 and Table 5). Seed DNA extraction had higher yield than young leaf and old leaf respectively, showing how best RT-PCR would be suited in evaluation of the two loci from different tissues using the stated protocols.

Locus SB3420 was amplified from *S. bicolor* accessions yielding CP values of 23.46 cycles, 23.62 cycles and 23.12 cycles from young leaf tissues, seed and old leaf tissues respectively (Table 5). The expected melting temperature peaks of 85.34, 85.4 and 85.32°C were obtained from young leaf tissues, seed and old leaf tissues respectively. In *S. sudanense,* locus SB3420 yield CP values of 25.74 cycles, 24.84 cycles and 22.13 cycles from young leaves, seed and old leaves respectively. The locus was also amplified in *S. verticilliflorum* to yield CP values of 24.65, 21.73 and 20.60 cycles from young leaf tissues, seed and old leaf tissues respectively. In *S. halepense* the locus was amplified from the young leaf, seed and old leaf tissues to

obtain CP values of 21.29, 25.96 and 23.76 cycles respectively (Table 5).

Analysis of Real Time PCR efficiency in quantitative determination of crop alleles (SB3420 and SB1764) from different plant tissues of *Sorghum bicolor, Sorghum sudanense, Sorghum verticilliflorum* and *Sorghum halepense*

The CP values obtained were applied in quantifying the concentration of the original template in *S. bicolor*, *S. sudanense*, *S. verticilliflorum* and *S. halepense* samples. A standard curve demonstrated the increase in fluorescence at 530nm with the increase in concentration of DNA (Figure 8a). A regression, standard curve of $Y= -0.4295X+32.95$ with an R^2 of 0.929 was applied to determine the amount of starting template in nanograms (Figure 8b). The results revealed that young leaf tissues had starting template concentrations of between 16.8ng to 27.1 ng based on the data (Table 6) from locus SB3420, seed tissues had concentration of 18.9 ng to 26.1 ng while old leaf had concentrations of between 20.6 ng to 28.8 ng (Table 6). Locus SB1764,

Table 7. Sensitivity of Real Time PCR in detection and characterization of sorghum wild and weedy species sampled from crop sorghum growing AEZs in Western Kenya.

AEZ	SB3420			SB1764			SB3258		
	CP	TM1°C	TM2	CP	TM1°C	TM2	CP	TM1°C	TM2
LM₁	22.77	82.70	85.32	27.98	87.59	-	20.64	82.20	-
LM₁	22.28	85.58	-	24.85	87.75	-	21.48	82.11	-
LM₂	24.69	81.53	85.63	28.56	80.75	87.64	24.59	81.90	-
LM₂	23.46	81.50	85.34	24.95	87.84	-	23.56	82.33	-
LM₂	22.11	81.86	85.58	29.84	87.63	-	27.20	81.72	-
LM₂	20.86	81.44	84.97	29.91	82.31	87.74	24.99	-	-
LM₂	24.84	85.07	-	30.10	81.59	87.38	22.88	81.56	-
LM₃	25.67	85.61	-	30.79	81.54	87.61	25.66	81.38	-
LM₃	25.74	77.71	81.06	24.02	87.84	-	23.64	-	-
LM₃	21.29	81.10	85.74	25.28	87.95	-	22.92	81.58	-
LM₃	24.90	81.07	85.35	26.22	87.84	-	21.28	82.18	-
LM₃	24.00	81.81	85.34	26.18	87.76	-	22.28	81.75	-
LM₃	22.92	83.53	-	27.62	87.72	-	20.25	82.24	86.97
LM₃	23.62	85.40	-	23.01	87.65	-	21.68	81.55	-
LM₃	25.96	85.42	-	21.90	87.79	-	20.95	81.93	-
LM₄	21.73	80.58	85.28	25.70	87.42	-	21.45	81.43	-
LM₄	23.76	85.23	-	29.73	87.40	-	20.58	82.31	-
LM₄	20.60	85.28	90.54	25.45	87.73	-	20.86	82.45	-
LM₄	22.13	81.11	85.13	29.16	81.11	87.38	20.89	82.60	-
LM₄	24.00	85.38	-	28.62	80.48	87.45	22.49	81.74	-
LM₄	23.07	85.28	-	29.90	87.46	-	20.51	82.42	87.33
S.B.	23.12	85.52	-	29.62	87.43	-	20.65	81.47	86.11

LM, Lower midland; S.B., *S. bicolor* +ve control; CP, crossing point; TM, melting peak.

showed concentrations of between 28.1 ng to 29.0 ng in young leaf tissues, 28.0 ng to 28.2 ng in seed and 28.0ng to 28.3ng in old leaf tissue samples. On locus SB1764 starting concentration was determined to be between 28.0 ng to 29.0 ng in all species and tissues. *S. bicolor* had 29.0 ng from young leaf as compared to 28.3 ng in *S. verticilliflorum* and 28.1ng in *S. halepense* (Table 6). Quantitative evaluation determined the starting concentration of template thus gave an indication to the copy number and amount of target DNA in the sample.

Analysis of the spontaneous occurrence of S. bicolor alleles in wild populations of sorghum collected from the three counties of Kenya using real time PRC

To analyse the spontaneous occurrence of *S. bicolor* alleles using RT-PCR, loci SB3420, SB1764 and SB3258 were determined in wild populations of sorghum and the melting point analysis for the loci from wild populations showed presence of alleles of both crop and wild origin (Table 7). In addition, analysis of wild sorghums populations from different AEZs showed their distribution in Western Kenya and the contribution of rare alleles from

S. bicolor in these populations (Table 3, 4, 5). The diversity in the populations resulting from evolutionary factors such as polyploidy, mutation and interspecific hybridization could have been important in the different AEZs.

Determination of crop alleles (SB3420, SB1764 and SB3258) from wild populations of sorghum

Presence of crop loci in the wild and weedy sorghum species was evaluated using RT-PCR. Three crop loci SB3420, SB1764 and SB3258 amplified differentially in the weedy sorghum (Figure 9, Table 7) collected from Homabay, Siaya and Busia counties and from four AEZs (LM₁, LM₂, LM₃ and LM₄). *S. bicolor* (BTX623) were used as a positive control while water was used instead of DNA template to form the negative control. Locus SB3420 in sorghum materials from LM₁ were amplified to yield CP values of 22.77 cycles and 22.28 cycles (Table 7). Weedy sorghums from LM₂ AEZs yield CP values ranging from 20.86 to 24.84 cycles (Table 7). Similar amplifications were demonstrated in materials from LM₃ which gave CP values ranging from 21.29 cycles to 25.96 cycles. Locus SB3420 was amplified from weedy sorghums
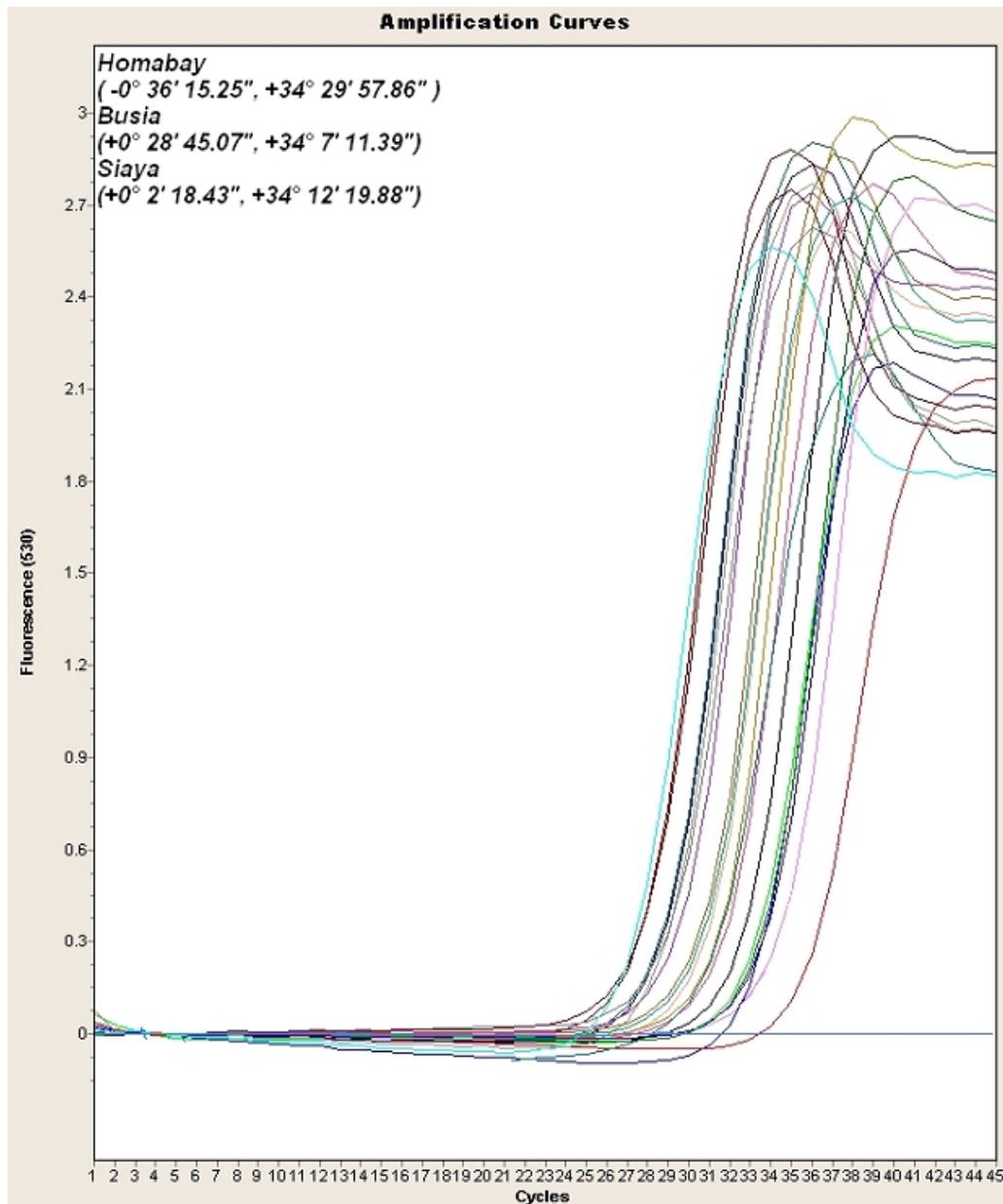
Figure 9. Real Time PCR amplification curves of crop specific locus (SB3258) found in the wild populations of sorghum collected from three counties.

collected from LM$_4$ zones and CP values ranged from 20.06 to 24.00 cycles (Table 7).

Locus SB1764 was amplified in the weedy sorghums from LM$_1$ giving CP values of 24.85 to 27.98 cycles, 24.95 to 30.10 cycles in LM$_2$, 21.90 to 30.79 cycles in LM$_3$ and 25.45 to 29.90 cycles in LM$_4$ (Table 7). Locus SB3258 from LM$_1$ had CP values of 20.64 to 21.48 cycles, sorghum from LM$_2$, had CP values ranging from 22.88 to 27.20 cycles while those from LM$_3$ exhibited CP values ranging from 20.25 cycles to 25.66 cycles. Samples from LM$_4$ showed CP values ranging from 20.51 to 22.49 cycles (Table 7).

Melting point analysis for loci SB3420, SB1764 and SB3258 from wild populations of sorghum

Melting peak analysis showed variations among the weedy sorghum species from the four AEZS; LM$_1$, LM$_2$, LM$_3$ and LM$_4$ (Figure 10 and Table 7). Real Time-PCR amplification of locus SB3420 gave TM peaks of between 82.70 to 85.58°C from samples in LM$_1$, between 81.44 and 85.63°C in LM$_2$, between 77.71 and 85.74°C in LM$_3$ and peaks between 80.58 to 90.54°C in LM$_4$. *S. bicolor* had a melting peak of 85.52°C (Table 7).

Locus SB1764 also had important variations among the
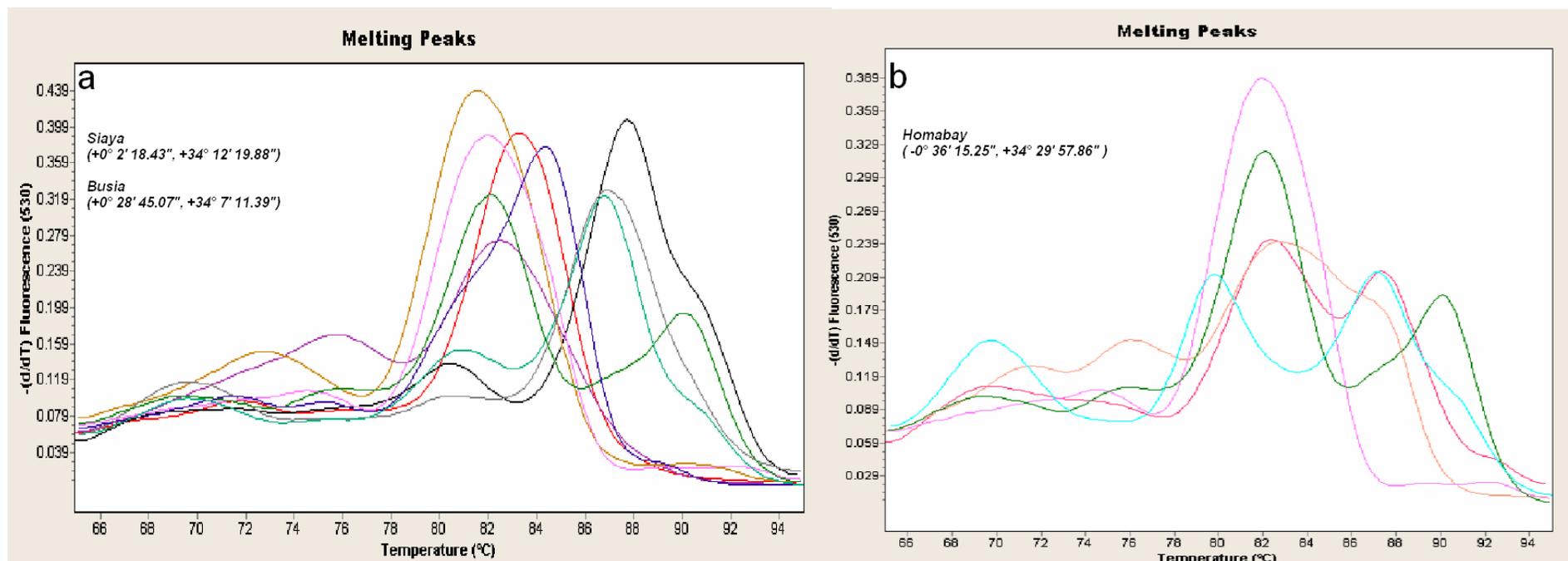
Figure 10. Melting curve analysis for weedy sorghum accession, showing variation in melting peaks from 69 to 92°C on locus SB3258 representing Siaya, Busia (a) and Homabay (b).

samples from different AEZS. Melting peaks of 87.59 and 87.75°C were obtained from LM_1 and in LM_2 peaks ranged between 80.75 to 87.84°C. Similar variability was also recorded in LM_3 where melting peaks ranged between 81.54 and 87.61°C. In LM_4 the weedy sorghums had melting peaks ranging between 80.48 to 87.73°C. *S. bicolor* showed a peak at 87.43°C (Table 7). Locus SB3258 was amplified in all the AEZS to give melting peaks between 81 and 88°C in all samples. *S. bicolor* had peaks at 81.47 and 86.11°C. LM_1 showed peaks ranging between 82.11 to 82.20°C; LM_2 had peaks between 81.56 to 82.33°C. In addition, LM_3 showed peaks between 81.38 to 86.97°C. LM_4 zone species

were analysed to yield peaks between 81.43 to 87.33°C (Table 7, Figure 10).

Inter-population analysis for wild sorghums populations in Sorghum growing AEZs in Western Kenya

Discriminant analysis for the weedy sorghums from LM_1, LM_2, LM_3 and LM_4 AEZs showed distinct population differences (Figure 11). Means of the four populations calculated from CP values and melting peaks were not significantly different. Their 95% confidence intervals showed significant overlaps. Sorghum populations from LM_1, and

LM_2 showed wider variation as compared with those from LM_3 and LM_4.

Low similarities were seen between LM_2 and LM_1 populations (1.3055) on Mahalanobis (D^2-) intergroup distance test (Table 8). Midway similarities were recorded between LM_3 and LM_2 (0.5304); LM_4 and LM_2 (0.6104) and *S. bicolor* and LM_2 (0.5099). High similarities were seen between LM_3 and LM_1 (0.193); LM_4 and LM_1 (0.1824); *S. bicolor* and LM_1 (0.2769); *S. bicolor* and LM_3 populations (0.1263). High similarities were seen when LM_4 and LM_3 populations were compared giving a D^2 value of 0.0865 and when LM_4 populations were compared with *S. bicolor* a D^2 value of 0.0364 was obtained (Table 8).
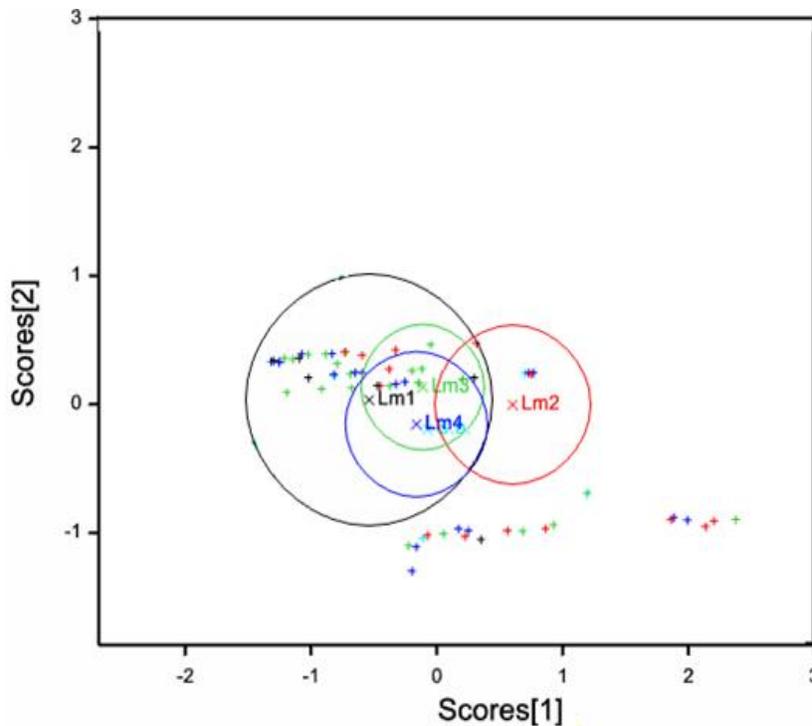
Figure 11. Discriminant analysis for weedy sorghums from LM$_1$, LM$_2$, LM$_3$ and LM$_4$ AEZs and a control *S. bicolor* (Sb) population based on crossing point and melting peak values. Circles are 95% confidence intervals around population means (N=50).

Table 8. Mahalanobis (D-squared) intergroup distances calculated from crossing point and melting peak values among weedy sorghums from LM$_1$, LM$_2$, LM$_3$ and LM$_4$ AEZs and a control *S. bicolor* (Sb).

| AEZ	LM$_1$	LM$_2$	LM$_3$	LM$_4$	S.b.
LM$_1$	0				
LM$_2$	1.3055	0			
LM$_3$	0.193	0.5304	0		
LM$_4$	0.1824	0.6104	0.0865	0	
S.b.	0.2769	0.5099	0.1263	0.0364	0

Analysis of rare alleles from S. bicolor in wild accessions using Real Time PCR

Rare crop alleles from locus SB5458 in sorghum wild populations were evaluated using RT-PCR. Out of 22 samples the positive control (*S. bicolor*) and two samples from the weedy relatives were amplified to give the expected amplification curves (Figure 12a). Samples that did not have the alleles from the locus showed no amplification and were similar to the negative control. CP values were within the expected range with *S. bicolor* showing a threshold at cycle 23 while the other two samples exhibiting CP values at cycle 24 and cycle 33 respectively (Figure 12a). Melting curve analysis showed similar peaks for all the three samples amplified at 71°C. However the *S. bicolor* peak was three fold to six fold

longer than the other two amplifications from the wild populations (Figure 12b).The negative control and the rest of the wild sorghum samples that did not amplify did not show melting peaks.

DISCUSSION

DNA extracted from young leaf, seed and old leaf was successfully amplified on loci SB1764 and SB3420 using RT-PCR in crop and weedy species. Young leaf and seed DNA samples showed relatively lower CP value ranging from 20.51 cycles to 20.88 cycles as compared to DNA from old leaf that had CP value ranging from 20.81 cycles to 20.91 cycles on locus SB1764 (Figure 7). This was attributed to the low DNA yields from older leaves due to accumulation of compounds that limit DNA isolation. Differences among concentrations (ng) of starting template of *S. bicolor (2N=20)*, *S. sudanense (2N=20)*, *S. verticilliflorum 2N= 20)* and *S. halepense (4N=40)* as determined on loci SB1764 and SB3420 (Table 6) may be influenced by the size of PCR fragment (bp), number of alleles per loci, the ploidy status of the species and the origin of homeologous chromosomes. For instance loci SB3420 yield 580bp in *S. verticilliflorum* and 300bp in *S. bicolor* explaining the higher concentration of 28.8ng in *S. verticilliflorum* as compared to 22.9ng in *S. bicolor*. Young leaves and seeds tissues
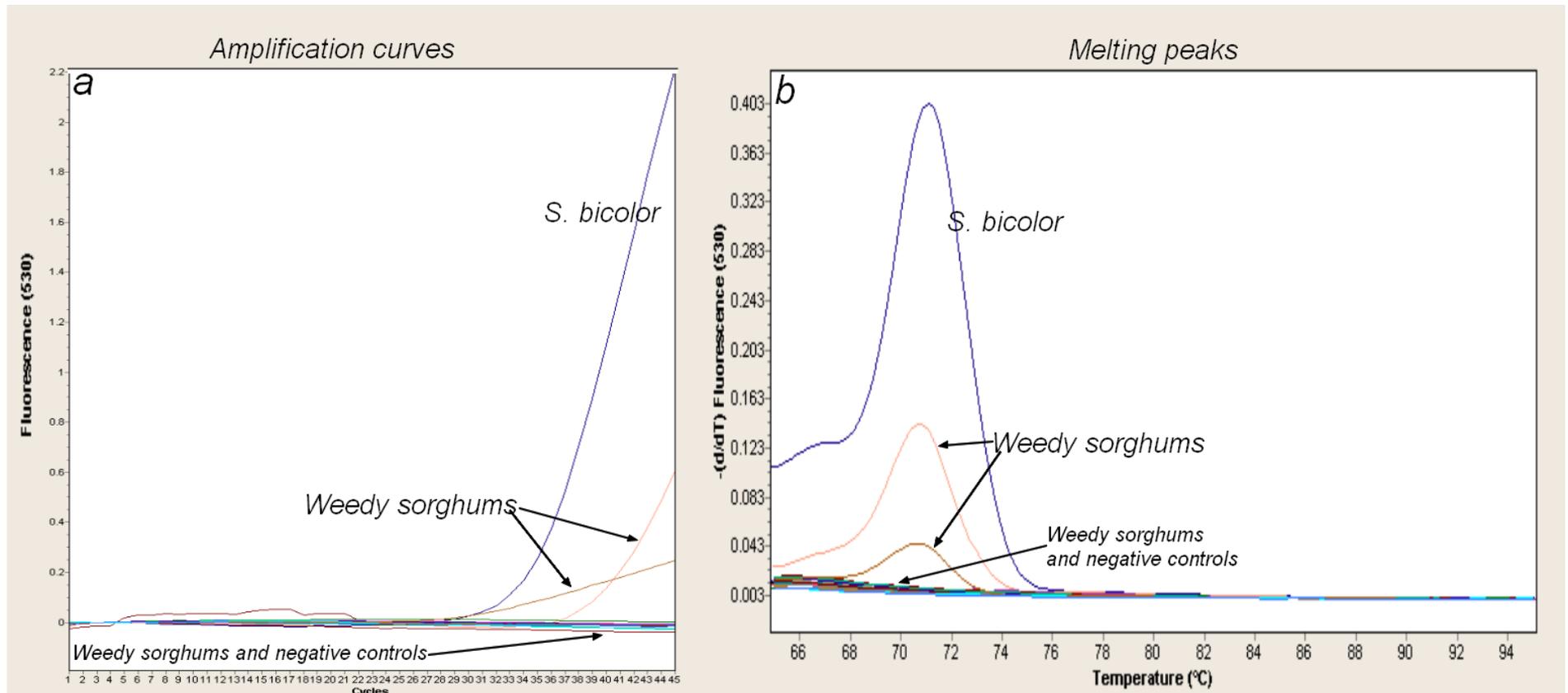
Figure 12. Real Time PCR amplification curves (a) and melting peak analysis (b) of rare crop specific loci (SB5458) from wild and weedy sorghum species showing a melting peak at 71°C.

have previously been used as source of template DNA in RealTime-PCR studies of transgenes in maize and soyabean (Hernández et al., 2004, Huang and Pan, 2005). The melting peak analysis demonstrated the allelic specificity of PCR products showing a peak of 87.57 to 87.91°C on locus SB1764 and 85.32 to 85.40°C on locus SB3420 in the different tissues. Similar melting peaks per locus irrespective of the tissue sampled from a given species were due to base similarity

of the amplified DNA fragments, which should always occur if the tissues are from the same samples.

RealTime-PCR amplifications were obtained on loci that possessed the specific SSR primer annealing sequences in both crop and weedy acessions. Locus SB3420 was amplified in *S. bicolor*, *S. halepense*, *S. sudanense* and *S. verticilliflorum* and thus RT-PCR was effective in qualitative determination of crop alleles in both

crop and weedy backgrounds for this locus (Figure 2). The loci had different alleles in the wild species that were differentiated through melting analysis. Real Time-PCR also determined absolute and relative quantification of alleles successfully in all the species by comparing the CP values. Templates with lower dilutions in all species had higher CP values than those with higher dilutions (Figure 3a, b). However variations on CP values were observed within and among the species,

For instance in Table 4, *S. halepense* had the least CP value of (31.21 cycles) in locus SB1764 as compared to *S. verticilliflorum* (32.51 cycles), *S. bicolor* (32.96 cycles) and *S. sudanense* (35.96 cycles). This could be due to the polyploidy nature of *S. halepense,* where the homeologous allele has maintained the features of the allopolyploidy segment (Tang and Liang, 1988). Therefore, providing more templates with the primer annealing sites and thus lower CP values. However in locus SB3258 *S. halepense* had a CP value of 22.88 cycles which was higher than *S. bicolor* (21.45 cycles) and *S. sudanense* (20.25 cycles). This locus could be within the allopolyploid segment that may possess different primer anneling sites leading to less amplification and higher CP values. These variations could also be attributed to the original concentration of the template DNA and the length of the PCR product. For instance, the low CP values from *S. verticilliflorum* (30.84 cycles) in locus SB3420 could have been due to higher starting concentrations, slightly different PCR fragment or differences in specificity of primer annealing sites. Previous studies have applied the RT-PCR technique in determination and quantification of alleles in maize and sorghum (Hernandez et al., 2003; Hernandez et al., 2004; Bedell et al., 2005; Pla et al., 2006; Yang et al., 2005).

The melting point analysis procedure was valuable in the determination and discrimination of SSR alleles from different species (Figure 5; Figure 6; Table 4). Evaluation Locus SB1764, SB3420 and SB3258 in *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* showed useful and consistent variations in the melting peaks. However, it was difficult to characterize SSR alleles within a species using melting peaks analysis as there was apparent limited variation within the species as compared to the variations among species on the loci studied. These conserved loci would be important while designing effective tools to measure gene flow. Melting temperature ranged between 83.29 to 88°C on locus SB1764 and between 86.01 to 80.88°C on locus SB3420 in the four species. Melting temperature analysis was able to determine the alleles from different species due to the differences in the base composition of the PCR products. Fragments with higher concentration of bases G/C had higher melting temparatures than those with higher A/T concentrations. In addition, alleles with differences in the PCR products melted at higher temperatures. The melting characteristics resulting from conservation of alleles in mutating and evolving species through concerted evolution (Paterson et al., 2009) could have enhanced the ability of the RT-PCR in differentiating sorghum accessions.

The capacity of the RT-PCR to differentiate PCR products and identify primer dimer artifacts by melting analysis has been previously demonstrated (Ririe et al., 1997). Melting point analysis of RT-PCR fragments has also been demonstrated in sorghum *tb2* and *kafarin* genes (Bedell et al., 2005). Hernandez et al. (2004)

applied melting point analysis in evaluating specific quantitative detection of transgenic maize event GA21. Melting analysis has been previously applied in the analysis of products of multiplex RealTime-PCR especially while amplifying distinct loci with specific alleles (Gachon et al., 2004; Hernandez et al., 2003; Huang and Pan, 2005).

Natural occurrence of alleles of crop and wild origin were demonstrated in the wild sorghum populations growing in sympatry with crop cultivars. Crossing point (due to polyploidy in *S. halepense*) and melting point analysis of SSR loci (SB3420, SB1764, SB3258 and SB5458), showed the presence of crop and rare alleles in wild sorghum populations. CP values ranging from 20.25 cycles to 30.79 cycles were observed on wild sorghums from LM_1, LM_2, LM_3 and LM_4 AEZs. The ten cycle difference on the CP values from the AEZs could be due to the polyploidy nature of material of *S. halepense* origin and the differences in original template concentration having an exponential effect on the differences. The difference may also be due to the quality and quantity of the extracted DNA. Melting peaks ranging from 69°C to 92°C were seen on locus SB3258. This demonstrated the presence of alleles from *S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum* with peaks at 88.00, 84.09, 84.65 and 83.92°C. The overlaps in melting point analysis among the species could be attributed to interspecific hybridization. Other uncharacterized alleles were also seen in the wild populations. Rare sorghum alleles were amplified in some members of the wild accessions collected from Western Kenya (Figure 12) showing the sensitivity of the technique and its ability to track exotic alleles in wild sorghum populations. This concurs with previous results by Pla et al. (2006) in which RT-PCR was applied in identifying isolated long distance gene flow from transgenic maize Mon810 into conventional cultivars in the field. Wild sorghum populations from the different AEZs from Western Kenya were differentiated using discriminant analysis and Mahalanobis (D-squared) intergroup distances. Populations from the different AEZs did not show significant differences (Figure 11) which could be due to seed distribution patterns that transcend AEZs. Crop sorghum seed is sometimes contaminated with common weed seed from the sorghum family, such that sharing these seed leads to ploriferation of the weedy species. Previous studies by Mutegi et al. (2011) demonstrated that farmers maintained some weedy species in sorghum fields. This would lead to seed mixing and sharing of weedy seeds. Similarities in selective weeding practices also tend to increase the frequency of certain weeds across the AEZs. In addition interspecific hybridization may have lead to the similarities of weedy species across AEZs. However, the intergroup distances showed higher similarities among AEZs around the lake (LM_4 and LM_3), that diminished to show wider differences towards AEZs away from the lake. This could be attributed to lake transport, such that communities around the lake share

more seed amongst themselves than they could with those further away. In addition the conditions around the lake may favour the proliferation of certain weedy species (Farm management hand book of Kenya, 2009).

CONCLUSIONS AND RECOMMENDATIONS

RealTime-PCR was useful in qualitative and quantitative determination of crop alleles in weedy sorghum. Melting analysis discriminated the species based on the allelic differentiation on specific loci. RealTime-PCR of DNA from young leaves, old leaves and seeds was effective in evaluating the presence of specific crop loci in weedy sorghums. Melting peak analysis of SSR loci SB3420, SB1764, SB3258 and SB5458 showed the presense of crop alleles in the wild sorghums. The study shows the potential for use of RealTime-PCR in evaluation of seeds and other plant materials in transit at entry points and on populations of growing plants in fields of cultivated and weedy sorghums for exotic genes including transgenes. In addition, the technique can be applied to analyze the number of copies of specific exotic genes in the target or non-target populations. Expression and transcription of such genes in the target or non target plants can also be quantified using RT-PCR.

Conflict of interests

The authors did not declare any conflict of interest.

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