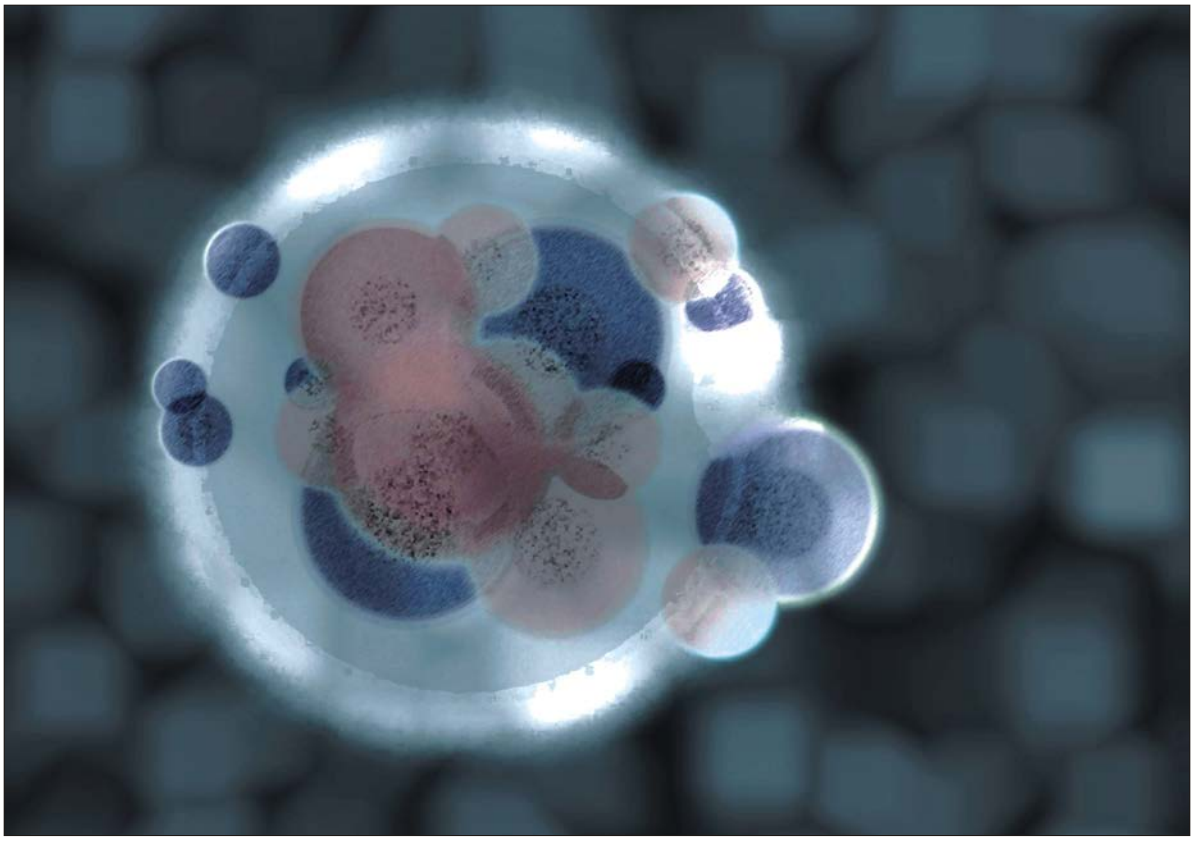

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Evaluation of Multiplex PCR in Detection of Crop Alleles and Differential Hybridization among Weedy Sorghum Populations

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Abstract: Utility of the PCR methodology in detection of crop alleles in weedy species has the potential for improvement through techniques that improve efficiency and minimize the cost and time required. This study evaluated the multiplex PCR procedure in concurrent detection of multiple crop alleles in wild sorghum populations and interspecific hybridization. Crop loci were amplified from seed and leaf samples of *S. halepense*, *S. bicolor*, *S. sudanense*, *S. bicolor* ssp. *verticilliflorum* open pollinated populations. Simultaneous amplification of combinations of loci SB1764, SB3420, SB5058 and SB5458 in the accessions gave the expected DNA banding profile. Loci combinations involving SB1764/SB5058, SB3250/SB1764 and SB1764/SB3008/SB5058 were important in determining polymorphism and interspecific hybridization within these species. Multiplex PCR reduced 2 loci and 3 loci assay time from 31-15.5 and 46.5-15.5 h, respectively. Multiplex PCR was useful in evaluation of the parental, F_1, F_2 and BC_1. Densitometric analysis of PCR fragments showed that amplifications from 35-50 ng of template had the best yield. *Sorghum sudanense* had higher affinity towards hybridization with the crop (45-76%) as well as the weedy materials (59-61%). Therefore, *S. sudanense* and its interspecific progeny seem to be an important bridge species in the sorghum genus.

Key words: Multiplex PCR, interspecific hybridization, sorghum, SSR loci

INTRODUCTION

The utility of sorghum in tropical agriculture for food, feed and biofuel production has necessitated the use of conventional genetic and transgenic approaches to improve yield and reduce its susceptibility to several biotic and abiotic stresses. However, there is a potential risk that these engineered genes will be transferred by pollen to wild relatives whose hybrid offspring will then become more aggressive or invasive. Quick, accurate and cost effective methods for identifying transgenes both in crops and in the wild backgrounds are necessary for containment and control of unintentional gene flow. Several modifications of the PCR technique are common place in tracking genes in crop and weedy populations. These modifications are based on the function and type of marker being applied. The markers include Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphisms (AFLP), Randomly Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Region (SCAR) and DNA Amplification Fingerprinting (DAF) (Kumar *et al*., 2009). Polymerase Chain Reaction (PCR) applications are developed, mostly based on the thermal cycling processes that connote a measure of improvement on the amplification efficiency, an aspect of cost and time efficiency, improved data analysis and provide for integrated automated systems. Examples where these innovations have been exploited in PCR applications include the use of loop-mediated isothermal amplification (Okuda *et al*., 2005; Wang *et al*., 2013), cycleave isothermal and chimeric primer-initiated amplification of nucleic acids (Urasaki *et al*., 2008), nested-PCR (Fang *et al*., 2004), real time PCR (Li *et al*., 2008; Heid *et al*., 1996) and multiplex PCR (Zangenberg *et al*., 1999).

Multiplex PCR techniques are developed for simultaneous amplification of two or more loci in genotypes of interest. The technique offers significant time and cost saving aspects when working with a large population or when assaying several loci

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(Zangenberg *et al*., 1999). Using one tube to amplify several loci reduces the chance of contamination or sample mixing and minimizes the amounts of reagents required in PCR reactions (Zangenberg *et al*., 1999). The technique yields several bands or data points on agarose gel electrophoresis, thus providing a functional platform for evaluating polymorphism within populations. The efficiency of simultaneous amplifications of several loci in a single reaction tube largely dwells on the primer design (Kebelmann-Betzing *et al*., 1998; Henegariu *et al*., 1997). First, the primers should have similar melting points with a plus or minus 1°C and be without stable secondary structures (hairpin structures). Secondly, the targeted product should not exceed ~300 bp where possible so as to reduce preferential amplification of shorter fragments (Sint *et al*., 2012). In an effort to reduce excess banding on agarose gels that may complicate analysis there is need to consider primers with two or less alleles in the population. The primers need to be devoid of 3' sequence complimentary overlaps to avoid primer dimer artifacts (Kebelmann-Betzing *et al*., 1998; Henegariu *et al*., 1997; Sint *et al*., 2012). These primer requirements are routinely integrated in primer design software (Rozen and Skaletsky, 2000; Kalendar *et al*., 2009).

Multiplex PCR has shown significant utility in differential amplification of plant and taxa specific chloroplast DNA (trn T-F cpDNA). The technique was successful in differentiating Poaceae and Apiaceae plant families (Wallinger *et al*., 2012). Multiplex PCR is useful in plant pathology in situations where more than one pathogen is important in a given plant population. The technique has been used in the simultaneous detection and differentiation of *Podosphaera xanthii* and *Golovinomyces cichoralearum* in sunflower (Chen *et al*., 2008). It has also been used in the evaluation of commercial F_1 hybrids and F_2 populations to identify plants that are homozygous or heterozygous for genes that confer resistance to tomatoe yellow leaf curl disease and root-knot nematode respectively (Chen *et al*., 2012). The technique has been previously used to evaluate genetically modified plants with multiple transgenes in soybean (Round up ready), maize (event176, Bt11, Mon810, T14/25) and canola (GT73, HCN92/28, MS8/RF3, Oxy235) (Hernandez *et al*., 2003). Cantu-Iris *et al*. (2012) applied multiplex PCR in detection of three transgenic events (Cry 1Ab, epsps and als) in maize, soybean and cotton.

Multiplex PCR has also been improved for real time PCR platforms and applied in detection of genetically modified maize MON810 and NK 603 (Huang and Pan, 2004). Multiplex PCR using microsatellite markers has shown value in evaluating diversity and population density of *Ardisia crenata* (Zeng *et al*., 2012) and several crops (Liu and Wu, 2012). This study hypothesized that multiplex PCR is an efficient tool in the detection of multiple crop alleles in wild populations. The multiplex PCR was used to evaluate interspecific hybridization among sympatric sorghum species (*S. halepense*, *S. bicolor*, *S. sudanense*, *S. bicolor* ssp. *verticilliflorum*). Codominant crop specific loci were designed and utilized to determine geneflow from crop to wild sorghums. In addition, the technique was applied to determine the differences in hybridization among crop and wild sorghums in order to analyse the role of bridge species in enhancing interspecific hybridization in the species.

MATERIALS AND METHODS

Growing of sorghum accessions in the field: The *S. bicolor*, *S. sudanense*, *S. halepense* and *S. bicolor* ssp. *verticilliflorum* seeds were obtained from the USDA germplasm bank selfing blocks. The populations were self pollinated in the greenhouse at the College of Agriculture and Veterinary Sciences (CAVS) (-1°14'59.72", +36°44' 30.79") for two generation. They were then evaluated for homozygosity using codominant SSR loci. The evaluated accessions of sorghum were grown at three sites (CAVS-1°14' 59.72", +36°44' 30.79"; Western Kenya at Alupe +0°28' 45.07", +34°7' 11.39" and Kakamega +0°12' 39.50", +34°57' 34.11"). The position of the pollinators relative to the weeds in concentric and alternating designs, staggered flowering period and wind direction were considered in laying the fields. Routine agricultural practices were applied during seeding, top dressing, weeding, pest control, bagging and harvesting.

Typical field conditions were simulated in the Alternating Flow Design (AFD). In the design *S. sudanense*, *S. halepense* and *S. verticilliflorum* were grown in alternating rows with *S. bicolor* at a spacing of 0.3 m within rows and 0.5 m between rows. These conditions involve a situation where weedy relatives are found in alternating rows with the crop sorghum. The hybridization between crop and weedy sorghums was estimated in typical agricultural field conditions. Planting was staggered to allow for synchrony in flowering of the experimental species; *S. sudanense*, *S. halepense* and *S. bicolor* ssp. *verticilliflorum*. All pollinations were allowed to occur naturally (Table 1). The experiment was set in a Randomized Complete Block Design (RCBD) with three replications at the CAVS. The recommended DAP (20-40 kg ha^{-1} of N) was applied at transplanting, top dressing with CAN was applied at 50-80 kg ha^{-1} of N. The plots were sprayed to control insect pests and bagged after natural pollination to keep off birds. At CAVS

panicles of each of the three weedy species and the crop were harvested. Six weedy species panicles and 2 crop species panicles per row were sampled; seeds were dried, threshed and stored in labeled packets in greenhouse conditions.

Concentric field designs were sited on two plots of 210 m in diameter to estimate the differences in interspecific hybridization among sorghums. The two fields were set in Western Kenya at Alupe (+0°28'45.07", +34°7'11.39") and Kakamega (+0°12'39.50", +34°57'34.11") and utilized to study the differences in interspecific hybridization within the sorghum species. The *S. bicolor*, *S. sudanense*, *S. halepense* and *S. bicolor* ssp. *verticilliflorum* were sown and staggered to allow for synchronized flowering. Recommended DAP (20-40 kg ha^{-1} of N) was applied at sowing and top dressing with CAN was applied at 50-80 kg ha^{-1} of N. Panicles of each of the three weedy species and the crop were harvested from Alupe and Kakamega and transported to CAVS where they were dried, threshed and stored.

Allele selection, primer design and multiplex primer combinations: Primers were designed, based on the polymorphic SSR sequences (Table 2). Simple Sequence Repeat (SSRs) regions were identified from the sorghum genome (Sorbi1) published on the NCBI GeneBank and defined sorghum SSRs. The physical mapping data was obtained from Phytozome database (phytozome-*Sorghum bicolor* v1.4). The Simple Sequence Repeat Identification Tool (SSRIT) and the primer 3 programme were used to identify the di, tri and tetra nucleotide simple sequence repeats on the mapped probes and the flanking primers. The primer sequences were sent and synthesized by Invitrogen™ on a 25 nmol scale of synthesis. The primers obtained were re-suspended in nuclease free water to make a stock solution of 100 μM. These were stored at -20°C in functional aliquots until PCR.

Primer annealing sites were confirmed by using the BLAST procedure against the NCBI GenBank database. The primers were selected, based on their polymorphism and their ability to distinguish several wild sorghum accessions. Primers were also designed to have similar annealing temperatures for high specificity. In addition, the primers were designed to exclude 3' and 5' stable structures and complimentary sequences with other primers used in the multiplex reaction. The PCR loci were combined in multiplex reactions based on lack of sequence complimentarity in the primer sequences and similar melting temperatures (Table 3). Multiplex PCR involving two loci and three loci were evaluated in the study.

Preparation of samples and DNA extraction: Two to five week old leaves from parental genotypes namely *S. bicolor*, *S. bicolor* ssp. *verticilliflorum*, *S. halepense*, *S. sudanense* and their F_1 populations were washed with

Table 1: Anticipated crosses between crop sorghum and wild sorghum species in open uncontrolled pollination

Cross
S. halepense×*S. bicolor*
S. bicolor×*S. halepense*
S. sudanense×*S. bicolor*
S. bicolor×*S. sudanense*
S. bicolor ssp. *verticilliflorum*×*S. bicolor*
S. bicolor×*S. bicolor* ssp. *verticilliflorum*

Table 2: Loci and primer sequences applied in the study

Marker name	Forward primer (5'→3')	Reverse primer (5'→3')
SB3258	ATTGTTGTCCTCCCCTCCACC	AGTACCTGAACCAGGCGTCGCT
SB3806	TGGAGCGAGGATGTAAGATCTGTG	CCAGCCACACAATTAAGACCCAAT
SB3978	CTGGTGAAGGTTGAAGAAAGGGTC	GCCACAAACATAAGGGGTTCACTC
SB4688	CTGTAAGCATGATGAAGGTCGTGG	AAGAAGGTGATGACAGGGATGGAG
SBI000X	CTAGAGGATTGCTGGAAGCG	CTGCTCTGCTTGTCGTTGAG
SB1764	CTTGTGCTTGCTTGCACCATATTC	GTCGATGAGGAGCTTCATGCTCAG
SB3420	GAGCCAGCATGCATGATAATTGTT	CACAAAGGCATGACAGTCAATCAA
SB5058	GAGAATTGGAAGAAAGCCTCGGTT	CAGAGCTCCTAAACGGTCCTCAAA
SB5458	AATGTGGTGGTGTTGTGTCTCCAT	TCTACTGCTATCATCGCCTCCACC

Table 3: Melting temperature and expected fragments from SSR loci combinations applied in multiplex PCR for the sorghum accessions

Loci combination		Primer melting temperature		Expected bands (bp)	
Primer 1	Primer 2	Primer 1	Primer 2	Primer 1	Primer 2
SBI000X	SB1764	F64/R64	F65/R66	280; 290	300; 850
SBI000X	SB3420	F64/R64	F64/R64	280; 290	300
SB1764	SB3420	F65/R66	F64/R64	300; 850	300
SB3420	SB5058	F64/R64	F65/R65	300	250; 320
SB1764	SB5058	F65/R66	F65/R65	300; 850	250; 320
SB1764	SB5458	F65/R66	F64/R65	300; 850	300
SB3258	SB1764	F64/R66	F65/R66	280; 800	300; 850

NB/F and R-represents forward and reverse primers, respectively

soap in running water to remove dust particles and other debris and weighed. About 0.3 g of each sample was kept in clean and labelled polyethene bags on ice until grinding. In addition, seeds and old leaves collected from the counties were stored on ice and transported to the laboratory. Old leaves were washed and weighed, while the seeds were dried and threshed before being weighed for DNA extraction. Genomic DNA was obtained from 2-5 week plants grown at CAVS, old leaves and seed obtained from the three sites. Genomic DNA from young leaves (from 2-5 week old plants), old leaves (from 5 week old and above) were extracted by using a modification of a CTAB extraction procedure as described by (Doyle and Doyle, 1990; Barnaud *et al*., 2008). The DNA from seeds was extracted from the samples by use of a modified CTAB-based DNA extraction protocol previously described by (Delobel *et al*., 2007) in maize. Agarose gel electrophoresis for genomic DNA extracted from young leaf, old leaf and seed tissues was done before running multiplex PCR. The multiplex PCR products were separated and analysed using a 4% UltraPure™ Agarose from Invitrogen™.

PCR reactions: Polymerase chain reaction was done in 0.5 mL reaction tubes with a hybaid® thermocycler. Reaction volumes of 11 μL were used in all experiments. The reaction conditions were set in conventional PCR format using Invitrogen™ PCR reagent system, Taq DNA polymerase with (W-1) Invitrogen™ and 10 mM dNTP Mix, PCR grade Invitrogen™. The annealing temperatures for the primer pairs ranged between 2-4°C (59-65°C for 30 sec) (Table 3) below each of their melting temperatures. The PCR denaturation and elongation temperatures were maintained at 94°C for 45 sec and 72°C for 90 sec, respectively and all programmes were set for 35 cycles. A multiplex approach was applied to amplify two or more loci simultaneously as shown in Table 4. Two to three loci were amplified in each tube with high annealing temperature of 64°C.

PCR products were evaluated on 3-4% UltraPure™ Agarose gels. Three to four grams of agarose (UltraPure™ Agarose, Invitrogen™) was added in 300 mL of 1×TBE buffer at room temperature and stirred to remove clumps. The agarose was melted by heating in the microwave oven till boiling. The 15-23 μL of ethidium bromide (10 mg mL^{-1}) was added after cooling to approximately 60-70°C. This was poured in gel casting equipment with 50 well combs and left to solidify, air bubbles were removed. Mineral oil was removed from PCR products and 1 μL of loading dye (BlueJuice™ gel loading buffer) Invitrogen™ was added to 11 μL of the PCR products including a 100 bp DNA ladder Invitrogen™ 1 μg μL^{-1}. These were loaded in the wells while the gel was submerged in electrophoresis buffer. Electrophoresis was done at 120 V for 2-4 h to ensure maximum separation of fragments with varying molecular weight. The gel was placed on a UV transilluminator and a photograph was taken.

Statistical analysis: Allelic polymorphisms were estimated in POPGENE version 1.31. Differences in interspecific hybridizations were evaluated through analysis of variance and species means were compared in GenStat 14. All amplifications were analysed on 4% UltraPure™ Agarose, Invitrogen™ and densitometry was done using calibrated molecular weight and concentration markers.

RESULTS

SSR loci polymorphisms among *S. halepense*, *S. bicolor*, *S. sudanense*, *S. bicolor* ssp. verticilliflorum and their crosses: Monoplex PCR gave a fragment of 300 bp from *S. bicolor* in all the 4 loci (SB1764, SB3420, SB5058, SB5458). However, in *S. bicolor* ssp. *verticilliflorum* there were larger bands of 490 bp from locus SB1764 while in *S. halepense* a band of 850 bp was obtained from locus SB3258 (Fig. 1). Using single loci (monoplex) PCR, members of the *S. bicolor*, *S. sudanense*, *S. halepense* and

Table 4: PCR reaction, concentrations of components in a 50X master mix and in the final 11 μL reaction volume

PCR component	Reaction concentration	50 sample master mix	Reaction volume in 11 μL
Nuclease free water		379.5 μL	7.59
10×Taq DNA polymerase reaction buffer	1X	55 μL	1.10
Magnesium chloride	1.5 mM	16.5 μL	0.33
10 mM dNTP	0.2 mM	11 μL	0.22
W1 detergent	0.05%	27.5 μL	0.55
Primers	0.5 mM	2.75, 2.75	0.055
Primers	0.5 mM	2.75, 2.75	0.055
Primers	0.5 mM	2.75, 2.75	0.055
Primers	0.5 mM	2.75, 2.75	0.055
Primers	0.5 mM	2.75, 2.75	0.055
Primers	0.5 mM	2.75, 2.75	0.055
Taq DNA polymerase enzyme		11 μL	0.5 U
Template DNA	50-100 ng		0.8
Mineral oil overlay			25-30

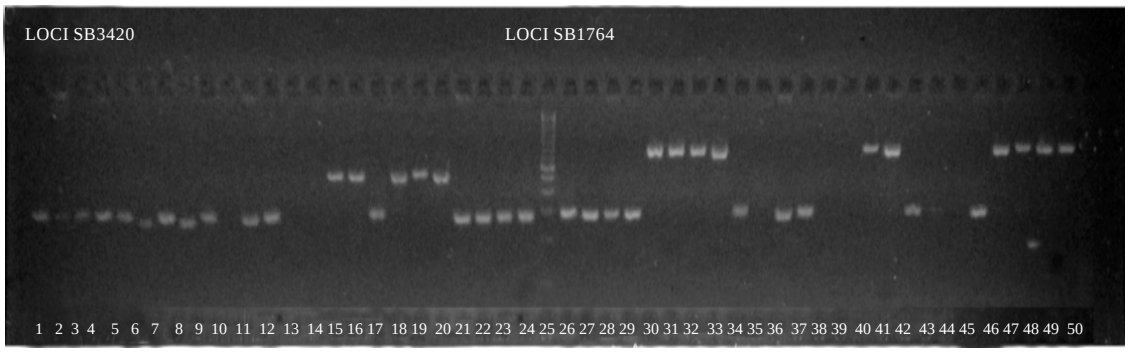

Fig. 1: The 4% agarose gel electrophoresis of monoplex PCR showing SSR loci polymorphisms among *S. bicolor* (lane 1, 2, 3, 4), *S. halepense* (lane 5, 6, 7, 8), *S. sudanense* (lane 9, 10, 11, 12), *S. verticilliflorum* (lane 13, 14, 15, 16) on loci SB3420 and *S. halepense* (lane 30, 31, 32, 33), *S. bicolor* (lane 26, 27, 28, 29), *S. sudanense* (lane 34, 35, 36, 37), *S. verticilliflorum* (lane 38, 39, 40, 41) on loci SB1764. Lane 25 contains the 100 kb ladder

Table 5: Fragments obtained from multiplex PCR applications on three species and their F_1 progenies using different SSR loci combinations

Loci combination	Sb alleles	Ss alleles	Sh alleles	Ss×Sb	Sh×Sb
	(bp)				
SBI000X; SB1764	290; 300	300; 300	280; 850	290; 300	280; 290; 300; 850
SBI000X; SB3420	290; 300	300; 290	280; 0	290; 300	280; 290; 300
SB1764; SB3420	300; 300	300; 290	850; 0	300; 290	300; 850
SB3420; SB5058	300; 250	290; 0	0; 320	300; 250; 290	290; 320
SB1764; SB5058	300; 250	300; 0	850; 320	300; 250	300; 250; 320; 850
SB1764; SB5458	300; 300	300; 0	850; 0	300	850; 300
SB3258; SB1764	800; 300	400; 290; 300	280; 850	800; 300; 400; 290	280; 850; 800; 300

NB: Fragment 580 originates from hybridization with *S. verticilliflorum* (Sv). Sh: *S. halepense*, Sb: *S. bicolor* and Ss: *S. sudanense*

S. bicolor ssp. *verticilliflorum* species were characterized and differentiated. Primer SB1764 gave a 300 bp fragment in *S. bicolor*, 300 bp fragment in *S. sudanense*, 850 bp fragment in *S. halepense* and 890 bp in *S. bicolor* ssp. *verticilliflorum* (Fig. 1). Primer SB3420 gave a 300 bp fragment in *S. bicolor*, 290 bp fragment in *S. sudanense*, no fragment in *S. halepense* and a 580 bp fragment in *S. bicolor* ssp. *verticilliflorum* (Fig. 1).

Simultaneous amplifications of alleles from different loci and genotypes through a multiplex PCR approach: Primers SB1000x in combinations with primers SB1764, SB3420, SB5058 and SB5458 had high resolutions on agarose gels and were effective in tracking crop alleles (Table 5). Combining primers for loci that exhibited polymorphism on the presence or absence of alleles gave significant resolutions as given in Table 5. The loci that had more than five alleles did not give distinct banding profiles in primer combinations.

Two loci combination in Multiplex PCR in amplification of SSR alleles from parental and F_1 populations: Two loci, used in multiplex reactions provided the most information on the alleles present in the species (Table 5 and Fig. 2). Combining primer SB1000x and primer SB1764 gave 300 bp fragment and 290 bp fragment in *S. bicolor* and *S. sudanense*, respectively. In *S. halepense* the amplified fragments were 850 bp long from SB1764 and 280 bp long from SB1000x (Table 5). In *S. bicolor* ssp. *verticilliflorum* a band of 890 bp long was recovered from SB1764, while 270 bp fragment from SB1000x was seen (Table 5 and Fig. 2). These differences were useful in simultaneous two loci identifications in the parents and the crosses. For instance, profiles of upto four DNA bands of 300, 290, 850 and 280 bp were recovered in a cross between *S. bicolor* and *S. halepense* (Fig. 2). This could be easily distinguished from each of the parents that had only two bands each.

Combining primers SB1764 and SB3420 showed high potential for tracking crop genes in wild sorghum species of *S. halepense*, *S. sudanense* and *S. bicolor* ssp. *verticilliflorum*. With combination involving primers SB1764 and SB3420 a 300 bp fragment was seen in *S. bicolor*; two fragments 300 and 290 bp in *S. sudanense*; and an 850 bp fragment in *S. halepense*. A 580 bp fragment from primer SB1764 was seen in genotypes that had *S. bicolor* ssp. *verticilliflorum* alleles (Table 5 and Fig. 2).

Populations of *S. sudanense* were differentiated from that of *S. halepense* and *S. bicolor* using the SB1764 and SB3420 primer combinations. The DNA banding patterns in the F_1 progenies exhibited two fragments 300 and

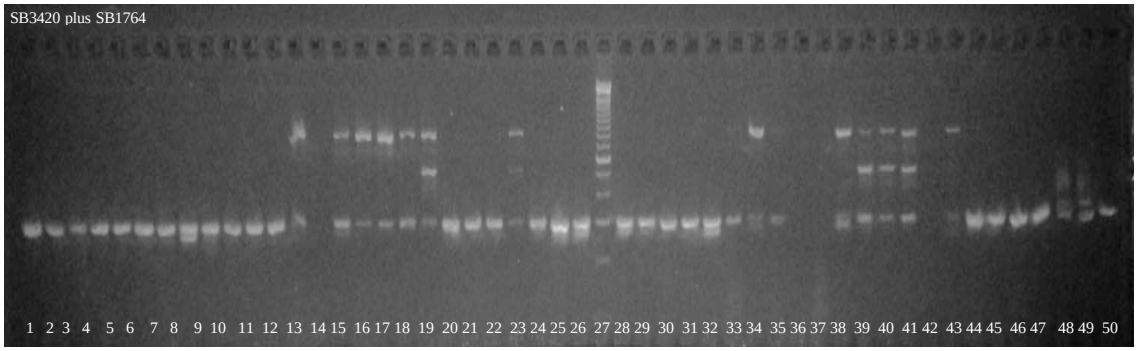

Fig. 2: Agarose gel electrophoresis showing fragments obtained from two SSR loci multiplex PCR applications; polymorphisms on loci SB3420 and SB1764 from *S. bicolor* (lane 1-6; 21-26), respectively, *S. sudanense* (lane 7-12; 28-33), *S. halepense* (lane 13-19; 34-39), *S. verticilliflorum* (lane 20; 40-45) and their field F_1 crosses (lane 9, 19, 23, 32, 34, 39, 40, 41). Lane 27 contains the 100 kb ladder

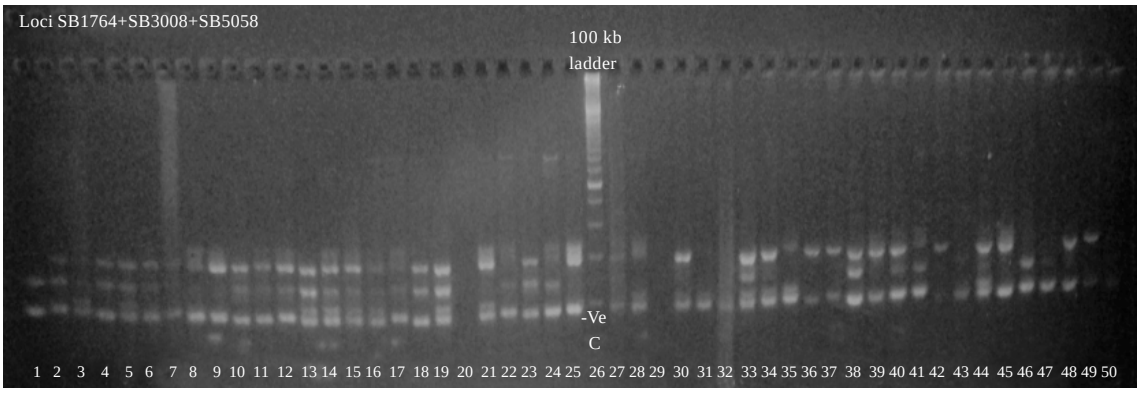

Fig. 3: Agarose gel electrophoresis showing fragments obtained from three loci (SB1764, SB3008 and SB5058) after multiplex PCR in *S. bicolor* (lanes 8-25) and *S. halepense* (lanes 33-41). Lane 26 contains the 100 kb ladder

290 bp in a cross between *S. sudanense* and *S. bicolor*, while two fragments 300 and 850 bp were seen in a cross between *S. halepense* and *S. bicolor*. A cross between *S. bicolor* ssp. *verticilliflorum* and *S. bicolor* had both the 300 and 890 bp fragments (Fig. 2). Combining primers SB1764 and SB5458 in multiplex reactions had little potential in differentiating *S. bicolor* from a cross between *S. bicolor* and *S. sudanense*. Both gave a fragment of 300 bp. However, the combination of primers SB1764 and SB5458 was useful in differentiating *S. bicolor* (300 bp) and *S. halepense* (850 bp) parents and the F_1 cross between *S. halepense* and *S. bicolor* (300 and 850 bp) (Fig. 2). A similar situation was seen in the cross between *S. bicolor* ssp. *verticilliflorum* and *S. bicolor* where two fragments (300 and 890 bp) were seen in the F_1 population.

Multiplex PCR involving three loci combination: Primers for loci SB1764, SB3008 and SB5058 amplified the expected fragments from the sorghum accessions (Fig. 3). Fragments of 250, 200 and 300 bp were amplified from *S. bicolor* while 320, 195 and 850 bp bands were amplified from *S. halepense* using these primers (Fig. 3).Combining three loci (SB1764, SB3420 and SB5058) or (SB1000x, SB5058 and SB3806) or (SB1764, SB3420 and SB5458) or (SB1000x, SB1764 and SB3420) did not yield the expected DNA bands on agarose gels. Combinations of three primers per reaction gave a 300 bp fragment in the samples collected from wild sorghum populations in Western Kenya and also in the three feral populations *S. halepense*, *S. sudanense* and *S. bicolor* ssp. *verticilliflorum*. The 300 bp band was only expected in *S. bicolor* accessions.

Use of Mulitplex PCR in evaluating F_2 and BC_1 populations obtained from interspecific hybridizations: Two loci multiplex PCR was applied in amplifying crop

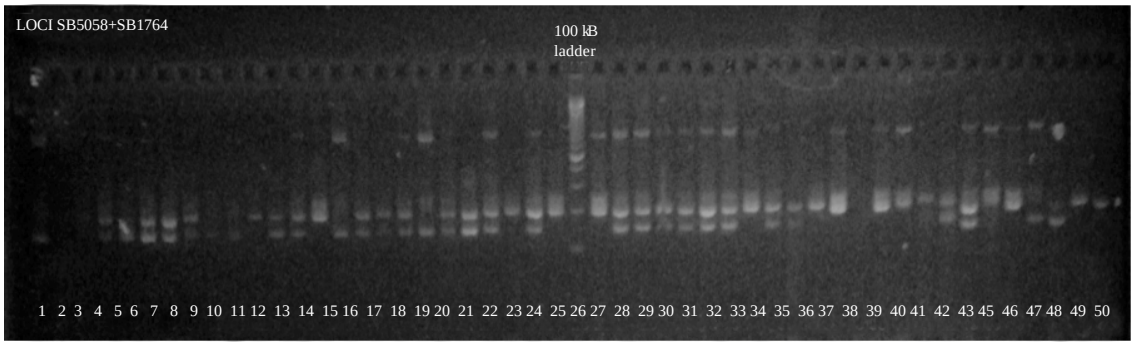

Fig. 4: Agarose gel electrophoresis showing two loci (SB5058 and SB1764) multiplex PCR products from F_2 population from *S. halepense*×*S. bicolor* (lanes 15-25), F_2 population from *S. sudanense*×*S. bicolor* (lanes 4-14), BC_1 populations (lanes 34 to 48). Lanes 49 and 50 represent *S. bicolor* and *S. sudanense* respectively. Lane 26 contains the 100 kb ladder

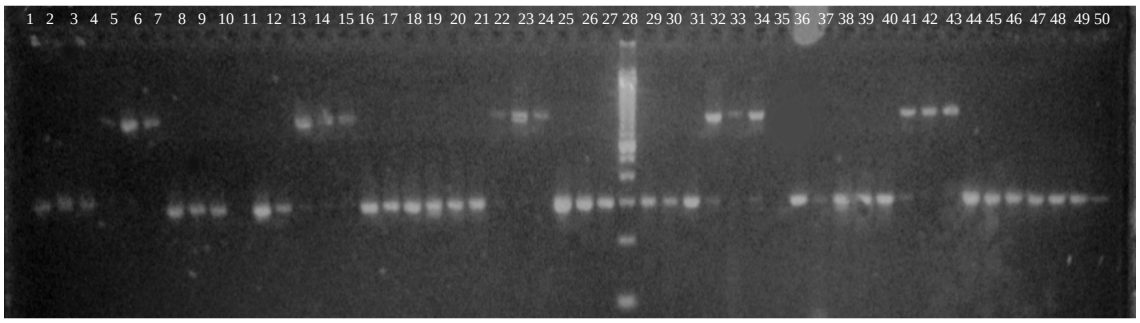

Fig. 5: Multiplex PCR (loci SB1764+SB3420) efficiency in template dilution sets of 1:1 (ln 10-18), 1:2 (ln 19-27), 1:3 (ln 29-37), 1:4 (ln 38-46) and controls (ln 1-9; 47-50) in three species *S. halepense*, *S. bicolor* and *S. sudanense*. Lane 28 contains the 100 kb ladder

Table 6: Mean weight of four multiplex PCR amplified fragments in nanograms (ng) (±SE) from diluted DNA templates (loci SB1764 + SB3420)

	PCR fragment weight (bp)			
DNA concentration (ng)	290	300	850	890
1 (~150)	47±5	28±4	30±9	37±9
1:1 (~75)	42±6	29±5	37±9	37±9
1:2 (~50)	63±6	51±5	33±9	37±9
1:3 (~37.5)	36±6	38±5	38±9	45±9
1:4 (~30)	58±6	52±5	45±9	50±9

alleles in the F_2 and BC_1 populations of *S. halepense*×*S. bicolor* and *S. sudanense*×*S. bicolor*. The expected alleles (300, 250, 850 and 320 bp) were seen in these segregating populations on the two loci SB5058 and SB1764 (Fig. 4). Lanes 15-25 represent the F_2 population from ♀ *S. halepense*×♂ *S. bicolor*, while lanes 4-14 represent the F_2 population from ♀ *S. sudanense*×♂ *S. bicolor*. BC_1 populations are shown from lanes 34-48. In some individuals one or more of the alleles were missing as seen in lanes 12, 17, 33, 41 and 46 (Fig. 4). Lanes 49 and 50 represent *S. bicolor* and *S. sudanense*, respectively.

Evaluation of optimum multiplex PCR template concentration: Dilutions of original template (~150 ng) had differences in the density of their respective PCR products. The differences of PCR product (fragment) and template dilution were significant ($p<0.05$) (Table 6). Similarly the variation of the PCR product weight (ng) from different species and dilutions was highly significant ($p<0.05$) (Table 6, Fig. 5). The 290 bp fragment had the highest density of 63 ng at 1:2 template dilution (~50 ng) while the 300 bp PCR fragment had a density of 51 ng at 1:2 template dilution but the highest was at 1:4 template dilution (~30 ng) with 52 ng. The other two fragments of

850 and 890 bp gave the highest resolution at 1:4 template dilution (~30 ng) at 45 and 50 ng PCR product, respectively (Table 6). All species had high product resolutions at template dilutions of above 1:2 (~50 ng). *Sorghum bicolor* showed high density with PCR products with yields ranging from 32-75 ng (Table 7). *Sorghum sudanense* had PCR products with yields ranging from 28-53 ng, while *S. halepense* had low product resolution of 22-44 ng (Table 7).

Multiplex PCR detection speed and costs: Upto 15.5 and 12.5 h were spent in multiplex PCR detection of loci from young leaf tissues and from seed tissues, respectively, in the accessions that were assayed. The use of extra loci in the multiplex PCR assays reduced the anticipated time by a significant proportion such that; half the amount of time required (from 31-15.5 h) was spent while using 2 loci and a third of the anticipated time was spent with 3 loci (from 46.5-15.5 h) (Table 8). Applying multiplex PCR reduced anticipated cost of using 2 loci and 3 loci by a half and two thirds, respectively (Table 9). The cost of assaying 100 samples (2010-2012 figures) averaged 347 US $. DNA extraction was valued at an average of 124 US $. PCR reagents at 180 US $ while using 100 reaction of 25 μL each and electrophoresis averaged 40 US $. Adding primers for the second or third loci in the reactions provided information on the added markers at the cost of one reaction (Table 9).

Table 7: Mean weight of multiplex PCR amplification products in nanograms (ng) (±SE) from diluted DNA templates extracted from three sorghum accessions

	Species		
DNA concentration (ng)	*S. bicolor*	*S. halepense*	*S. sudanense*
1 (~150)	45±4	22±4	37±5
1:1 (~75)	43±5	34±4	28±5
1:2 (~50)	75±5	33±4	50±5
1:3 (~37.5)	32±5	39±4	44±5
1:4 (~30)	65±5	44±4	53±5

Table 8: Time required for processes involved in SSR assay of plant genomic DNA from two tissues (leaf and seed)

	Time (h)	
Source of template process	Leaf DNA	Seed DNA
Genomic DNA extraction	7.0	4.0
PCR reagent preparation (50 Rxns)	1.0	1.0
Amplification in the thermocycler	4.0	4.0
Gel preparation	0.5	0.5
Loading PCR product in gel (50)	0.5	0.5
Running and analysis of PCR gel	2.5	2.5
Total	15.5	12.5

Table 9: Approximate cost for SSR locus assay of 100 samples (25 μL reaction) (2010-2012 costing)

Laboratory reagents	Cost for 100 samples US$
Genomic DNA extraction reagents	127
PCR reagents	180
Gel electrophoresis reagents	40
Total	347

N.B: Cost of labour, electricity, equipment depreciation and bench or laboratory space are not included

Differential hybridization in sorghum species was characterized using Multiplex PCR: Significant differences in interspecific hybridization were observed among the species (*S. bicolor*, *S. sudanense*, *S. halepense* and *S. verticilliflorum*) grown in UFD and AFD field designs ($p \leq 0.05$). Hybridization between *S. halepense*×*S. verticilliflorum* was recorded in both plots but at low frequencies (2.8%) (Table 10). A similar situation was seen with hybridizations between *S. sudanense*×*S. verticilliflorum* (2.6%), (Table 10, Fig. 6). Hybridization between *S. halepense*×*S. sudanense* and *S. verticilliflorum*×*S. sudanense* was recorded at 52% and 45% frequencies, respectively. The 48 and 45% hybrid seed were obtained from spontaneous field crossing among *S. sudanense*×*S. halepense* and *S. verticilliflorum*×*S. halepense*, respectively (Table 10). Crosses between *S. sudanense*×*S. bicolor* were the most frequent (60%) among all crops by weed hybridizations. *S. halepense*×*S. bicolor* hybridized at 55% while *S. halepense*×*S. verticilliflorum* hybridized at 43%. Site 2 had higher frequencies of hybridization among both weed to weed and crop to weed. Hybridization competition among the species planted in UFD plots was observed (Table 10, Fig. 6).

Table 10: Differences in spontaneous hybridization among open pollinated species in UFD plots

Spontaneous hybridizations		Hybridization (%)		
Female parent	Male parent	Site 1	Site 2	Both sites
S. halepense	*S. verticilliflorum*	4.3±1.0	1.2±1.0	2.8±1.4
S. sudanense	*S. verticilliflorum*	2.6±1.0	2.8±1.0	2.6±1.4
S. halepense	*S. sudanense*	76.0±3.4	34.1±3.4	55.2±4.8
S. verticilliflorum	*S. sudanense*	24.4±3.4	65.8±3.4	45.1±4.8
S. sudanense	*S. halepense*	43.8±3.4	45.9±3.4	44.8±4.8
S. verticilliflorum	*S. halepense*	24.4±3.4	65.8±3.4	45.1±4.8
S. halepense	*S. bicolor*	52.0±3.6	58.2±3.6	55.1±5.1
S. sudanense	*S. bicolor*	59.7±3.6	61.5±3.6	60.6±5.1
S. verticilliflorum	*S. bicolor*	47.5±3.6	38.7±3.6	43.1±5.1

Figures are means of spontaneous hybrids in 96 UFD plots in each of the two sites ±SED. Site 1: Samples from Alupe and Site 2: Samples from Kakamega

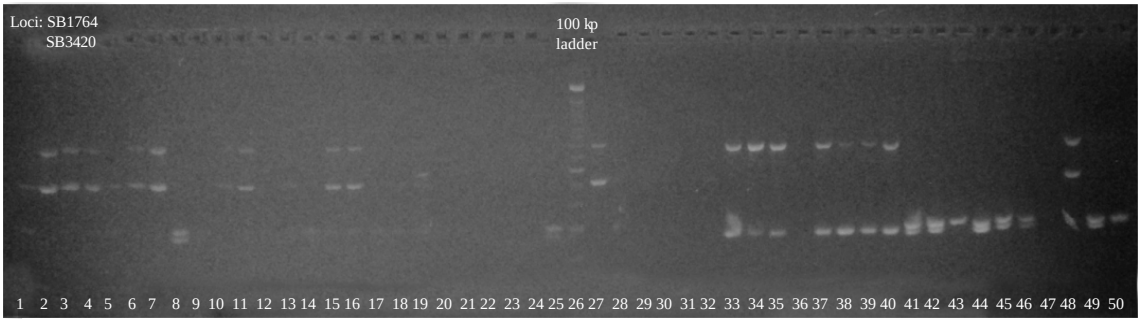

Fig. 6: Multiplex PCR on sorghum populations grown in open pollinated plots showing crop alleles (300 bp) and weed alleles (580, 850 bp) in other wild sorghums, NB. Lane 1-17 (*S. bicolor* ssp. *verticilliflorum* showing 850 bp and 280 bp from *S. halepense* and 300 bp from *S. bicolor*); Lane 17-40 (*S. halepense* showing 580 bp from *S. bicolor* ssp. *verticilliflorum,* 300 bp from *S. bicolor and S. sudanense*); Lane 41-49 (*S. sudanense* showing 580 bp from *S. bicolor* ssp. *verticilliflorum,* 300 bp from *S. bicolor*); Lane 50 (*S. bicolor*). Lane 26 contains the 100 kb ladder

DISCUSSION

Multiplex PCR was useful in simultaneous detection of loci and in allelic differentiation and determination of interspecific hybridization among members of the sorghum genus, their hybrids and subsequent generations in the study. Two loci combinations were useful when primers for loci SB1764/SB5058 and loci SB3250/SB1764 were used together (Table 5, Fig. 2). Simultaneous amplification of loci SB1764 and SB3420 in *S. bicolor*, *S. sudanense* and *S. halepense* gave the expected 280, 290, 300 and 850 bp after agarose gel electrophoresis (Fig. 5). These combinations showed 4 alleles and their differences among crop and weed sorghum accessions. Three loci combinations of SB1764, SB3008 and SB5058 showed biologically significant polymorphism among sorghum genus populations and their hybrids (Fig. 3). Multiple loci have previously been applied in multiplex analysis of genetic diversity of Eritrean sorghum landraces (Ghebru *et al*., 2002) and in determining genetic redundancy and diversity among orange accessions of sorghum (Dean *et al*., 1999). Loci positioned distal to the centromere on chromosomes had higher polymorphisms that loci that exhibited presence or absence of alleles and those had several alleles per loci showed polymorphisms that were useful in primer combinations. High resolutions were obtained on 4% PCR agarose gels stained with ethidium bromide and parental, F_1, F_2 and BC_1 populations were differentiated. Multiplex PCR was also useful in situations where weed accessions had null alleles in one loci accompanied with a distinct allele at another loci in the primer combinations (Fig. 3). Distinct crop alleles (transgenes) have been studied in soybean, maize and canola using a multiplex approach (Hernandez *et al*., 2003; Cantu-Iris *et al*., 2012). In previous studies multiplex-PCR was useful in evaluating genetic diversity, allelic analysis and discrimination in several crop and forage crops (Liu and Wu, 2012; Hernandez *et al*., 2003; Cantu-Iris *et al*., 2012).

The original template concentration had significant effect on the efficiency of multiplex PCR procedures. Densitometric analysis of the obtained PCR fragments showed that amplifications from 35-50 ng of template DNA had the best yield and thus resolution on agarose gels. This was observed in amplifications from the three species (*S. bicolor*, *S. halepense* and *S. sudanense*) used in the study (Table 7). Therefore, based on the resolution of the expected fragments and resolutions of PCR products from the different species a template dilution of 1:2 (~50 ng) to 1:5 (~30 ng) gave the best PCR yields (Table 7). The 290 bp fragment had high yields of 63 ng and 58 ng at a dilution of 1:2 (~50 ng) and 1:4 (~30 ng), respectively (Table 6). Similarly, high yields were observed at a starting concentration of 1:2 and 1:4 on the 300 bp fragment. In contrast, high yields were only observed at 1:4 (~30 ng) from the 850 bp and the 890 bp fragment (Table 6). This variation would be attributed to the fragment size and the competitive efficiency for utilization of PCR reagents in the synthesis of the shorter fragments (200, 300 bp) than in the longer fragments (850, 890 bp). Template dependent variation on the efficiency of multiplex PCR has been previously reported (Zangenberg *et al*., 1999; Sint *et al*., 2012). The base composition of the fragments would also be important in efficiency of amplification in multiplex PCR reactions. *Sorghum bicolor* had higher yield in multiplex PCR reactions than *S. halepense* and *S. sudanense* at dilutions of 1:2 (~50 ng) and 1:4 (~30 ng). This could be due to

species differences of the amplified fragments size and composition. Other studies (Zangenberg *et al*., 1999; Sint *et al*., 2012) show that the base composition of the fragments, the fragment size have an effect on the efficiency of multiplex PCR.

Using combinations of primers for the loci being assayed reduced the time required to complete PCR reactions by half every time a loci was added in the combination (Table 8). The time required for two or three loci multiplex PCR was comparable to using leaf tissues with monoplex PCR where a total of 15.5 h were spent from genomic DNA extraction to PCR and final electrophoresis. In the procedure, 45% of the total time utilized in detecting a target locus in the selection of accessions was spent on genomic DNA extraction. Using seed as a source of template, a total of 12.5 h were spent from genomic DNA extraction to final PCR (Table 8), genomic DNA extraction took 32% of the time. The cost of PCR was reduced due to shrinking the requirements of important and costly reaction components such as Taq DNA polymerase and dNTPs (Table 9). Previous study by Cantu-Iris *et al*. (2012) also demonstrates the potential of multiplex PCR in reducing the cost of genetic analysis especially in developing laboratories. The efficiency of multiplex PCR in tracking of crop alleles in weedy backgrounds was enhanced by using primers with similar melting temperatures of between 64-65°C (Table 3). However, combining more than three primer pairs reduced the resolution on the agarose gels. This situation could have been attributed to 3' complementary sequences among some primers involved in the reaction, complementary sections of PCR products and the increased need for the reagents necessary for the reaction mix (Zangenberg *et al*., 1999). Primer attributes, including primer size, melting temperature and base composition have effect on multiplex PCR efficiency (Zangenberg *et al*., 1999; Kebelmann-Betzing *et al*., 1998). In addition template concentration, reaction conditions and thermal cycler temperature programmes have previously been identified as important efficiency factors for multiplex PCR (Henegariu *et al*., 1997; Sint *et al*., 2012).

The frequencies of hybridization as obtained by multiplex PCR studies showed that *S. sudanense* had higher affinity towards hybridization with the crop as well as the weedy materials. This situation was true in both UFD sites (Table 10). Flower morphology and the vegetative nature of *S. sudanense* shows midway features between crop sorghum and most weedy sorghums including *S. halepense* and *S. verticilliflorum*. The higher hybridization frequencies in *S. sudanense* can be attributed to the origin of the diploid species (2n = 20) from a natural hybrid between *S. bicolor* and *S. arundinaceum* (Hacker, 1992). Therefore, maintaining floral features from both crop and weedy backgrounds. This may increase the potential for *S. sudanense* of hybridization among weedy and crop species. In addition the species has proven its utility as a forage crop especially when hybridized with *S. bicolor* (Hacker, 1992) and has been shown to naturally hybridize with crop sorghum (Pedersen *et al*., 1998). This hybridization is also seen in *S. verticilliflorum* which is a progenitor of Bicolor, Candatum and Kafir races and belongs in the subspecies Arundinaceum (NRC, 1996). *Sorghum halepense* is probably a disomic polyploidy or segmental allopolyploid (2n = 40). The species are found growing together in most sorghum production zones (De Wet, 1978; Duvall and Doebley, 1990). In most cases *S. sudanense* is propagated and maintained as a forage crop while *S. halepense* remains among the 100 worst weeds in the worldwide crop agriculture (Holm *et al*., 1977). High hybridization between *S. sudanense*×*S. bicolor*, *S. sudanense* by *S. verticilliflorum* and *S. sudanense*×*S. halepense* suggests that *S. sudanense* and its naturally existing hybrids may form a bridge for the genus. This may be important for species that have a growth habit that curtails hybridization with crop sorghums. The bridge species could hybridize with the crop and latter the weedy species that may not be interfertile with the crop resulting in increase in the frequency of either crop alleles in weedy populations. Multiplex PCR provides a quick efficient and less costly strategy, which can be applied in concurrent detection and identification of several alleles in bridge species that may hybridize with genetically distant species.

CONCLUSION

Concurrent amplification of two to three genes showed significant reduction in the time and cost required in PCR analysis. Analysis of allelic differences within the species and interspecific hybridization was cost effective and informative with the use of multiple loci per reaction. Precision in distinguishing species and study of crop alleles in interspecific hybrids and segregating sorghum populations was enhanced through concurrent multiple loci analysis. Template concentration, primer quality and reaction conditions were important in efficient amplification and analysis of multiple loci. The study recommends the use of multiplex PCR for the analysis of hybrids and subsequent introgressing populations for multiple exotic loci. The current trend in development of

crops with multiple transgene events and large minichromosome inserts encourages the development of versatile technologies to enhance detection in crops and their wild progenitors. Multiplex PCR can be applied in analysis of several genes on a transgene construct thereby giving valuable information on successful transformation and existence of breaks or recombination. Multiplex PCR can be applied in the ongoing analysis of crops with multiple transgene events and their interspecific hybrids with non-target wild and weedy populations.

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