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Full Length Research Paper

Comparison of two PCR-based DNA markers with high resolution melt analysis for the detection of genetic variability in selected quality protein maize inbred lines

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Molecular markers are fast, efficient and reliable in detecting distinct differences between genotypes at DNA level. In particular, polymerase chain reaction (PCR)-based markers are widely preferred for genotype characterization in diverse crop species, including maize. It is impossible, however, to obtain the entire information required in plant breeding and conservation programmes solely from a single marker system without integration with other technique(s). The current study was, therefore, initiated to compare results from random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers in combination with high resolution melt analysis for effectiveness in detecting genetic variability or similarity among selected QPM inbred lines. Eight fresh leaf samples (two per line) were collected from each line for DNA extraction. The genomic DNA extracts were subjected to polymerase chain reaction (PCR) and high resolution melt analyses which involved three RAPD primers and three SSR primer pairs using a Real-Time PCR System. The curves of the HRM analysis showed fairly similar melt patterns among the lines for both types of marker systems. Results obtained from the gel-electrophoresis and genetic distance measures for RAPD markers were also in agreement with the HRM analysis. In general, both marker systems in combination with HRM analyses were able to detect genetic variations within and between the inbred lines, thus justifying the potential use of HRM analysis and indicating the possibility of excluding the use of gel-electrophoresis at the early stage of screening of large number of samples of newly developed inbred lines in order to save time and resources.

Key words: Genetic variability, polymerase chain reaction, quality protein maize, random amplified polymorphic DNA, simple sequence repeats.

INTRODUCTION

Maize and wheat have been extensively exploited in genetic and cytogenetic studies compared to other cereal crops. Maize is one of the domesticated crop species with the highest level of molecular polymorphism. Nucleotide diversity of more than 5% has been reported at some loci of the maize genome (Henry and Damerval, 1997), and has been verified by high genetic variability both within and among maize populations as revealed by several genetic diversity studies. The molecular diversity

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of maize is approximately three to tenfold higher than that of other domesticated grass species (Buckler et al., 2001). Several factors have been suggested as reasons for the diversity in maize, including (1) variability of growing environments, domestication for various production systems and types of consumption preferences (Aguirre et al., 1998) have resulted in different types of maize in the world; (2) the predominantly out-crossing nature of maize has facilitated continuous gene exchange among maize populations, and in some cases, with their wild relatives; (3) the maize genome undergoes extensive chromosomal duplications, providing new mutational opportunities that lead to phenotypic variability (Helentjaris et al., 1998); and (4) transposons and retrotransposable elements have also played an important role in the creation of the wide variation among maize (Bennetzen et al., 2005).

Morpho-agronomic characters of crop plants have traditionally been used for germplasm identification. However, identification based on these characters is not efficient and reliable as they are highly affected by environmental factors. Since the late 1980s, different electrophoretic (Zillman and Bushuk, 1979; Tkachuk and Mellish, 1980) and reversed-phase high–performance liquid chromatography (RP-HPLC) (Marchylo et al., 1988; Scanlon et al., 1989) of seed storage proteins have been developed and are considered effective methods for cultivar identification. However, the ability of the techniques to discriminate among cultivars is limited. On the other hand, DNA-based molecular markers are breeding tools which are capable of providing high discrimination power (Perry, 2004). They are used in the identification of specific sequence variation between two or more genotypes and in many cases are more effective than biochemical assays (Lorz and Wenzel, 2008). Molecular markers are not influenced by environmental factors and are also fast, efficient and more sensitive than field evaluation for detection of large numbers of distinct differences between genotypes at the DNA level (Melchinger, 1999a). Several DNA marker technologies have been developed and are available for studying genetic variability. The choice of the most appropriate marker system greatly depends on the species, the objective of the marker analysis and the available resources (Lorz and Wenzel, 2008). PCR-based markers are widely preferred for genotype characterization in diverse crop species, including maize, as they are relatively simpler to use, non-destructible, requires smaller amount of DNA thus permitting many reactions from a single sample (Powell et al., 1996; Soleimani et al., 2002).

Hence, molecular markers such as RAPD, AFLPs and SSRs, are proposed to be an appropriate tool not only for breeding lines and hybrids (Bastia et al., 2001), and cultivars (Mohanty et al., 2001) but also facilitate the monitoring of introgression, mapping of quantitative trait loci (QTLs) (Paterson et al., 2003) and the assessment of genetic diversity (Warburton et al., 2002; Kassahun and Prasanna, 2003; Asif et al., 2005; Legesse et al., 2007; Pooja and Singh, 2011) in different crops including maize. However, it is impossible to obtain the entire information required in plant adaptation, breeding, evolutionary and conservation programmes solely from a single marker without integrating it with other technique(s) (Thormann et al., 1994). The current study was, therefore, conducted to compare the effectiveness of RAPD and SSR markers in combination with HRM analysis for detecting genetic variability or similarity among selected QPM inbred lines.

MATERIALS AND METHODS

Plant materials

Four QPM inbred lines coded as Q2, Q3, Q6, and Q7 with their pedigrees CML159, CML176, CZL01006, and Obatanpa-SRC1F3#-MALE, respectively, obtained from the African Centre for Crop Improvement (ACCI) maize breeding program at the University of KwaZulu-Natal were used for this experiment. They were planted in pots in the University's green house in March 2011. Each line was planted in two replicates. Two seeds per pot were planted and later thinned to one just 35 days after planting. Management and agronomic practices such as weeding, fertilizer application and watering were undertaken uniformly across all the lines as required.

Primer information

Three RAPD primers and three primer pairs of SSRs with sequence information described in Table 1 were used for the experiment. Since these markers had been selected for routine screening in the University of KwaZulu-Natal's molecular genetics laboratory, preliminary screening of SSR primers was not done.

Extraction of genomic DNA

Genomic DNA was isolated using the CTAB procedure. One-month old leaf samples of each line from the two replicates were collected and handled separately. Thus the total number of samples being analysed were eight. Leaf samples were collected by punching discs directly into the mini tubes using the lid of a microcentrifuge tube (1.5 ml). 400 µl of extraction buffer [200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% (W/V) SDS] containing proteinase K (125 µg/ml) was added into the tubes with leaf samples, and incubated at 37°C for 1 h. The leaves in the buffer were subjected to bead beater and 400 µl of CTAB solution [2× CTAB solution: 2% (W/V) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP (W/V) (polyvinylpyrrolidone) Mr 40,000] was added to the ground leaves. Then, the solution was vortexed vigorously for a few seconds and incubated in a water bath at 65°C for 10 min. Gentle extraction using chloroform : isoamyl alcohol (24:1) with 5% (V/V) phenol was carried out followed by centrifuging at 13,400 g in microcentrifuge at 4°C for 10 min and transfer of the supernatant to new tubes. Isopropanol with equal volume to the supernatant was added to the tubes after which the tubes were incubated at room temperature for 10 min to precipitate DNA. Centrifugation at 13,400 g was repeated for 5 min, the supernatant removed, and finally the DNA pellet was washed with 70% (V/V) ethanol, air dried, and re-suspended in 20 µl of TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8) for 5 h. DNA quantification was undertaken and absorbance of the concentrations was determined at 260 and 280 nm wavelengths using a NanoDrop

Table 1. List of primers used for the experiment.

Marker	SSR locus	Repeat types	Primer sequence
SSR	*Umc1243*	CAT	Forward: 5´ AACTGCAGAGTCGCCTGATCC 3´ Reverse: 5´ AAGCAGACTATGCTATGCTACGCC 3´
	Umc2390	TAC	Forward: 5´ GAAATGGCAGGGAAACTTGTTTAT 3´ Reverse: 5´ AAGAGGCAAGCAAGTGTACAGTGA 3´
	Bnlg2295	AG	Forward: 5´CGGAGGAGTGGTTCTTGAAA 3´ Reverse: 5´GGTTAGTGAAAGGGTTGCCA 3´
RAPD			OPA-12: 5´ TCGGCGATAG 3´ OPA-16: 5´ AGCCAGCGAA 3´ OPA-18: 5´ AGGTCACCGT 3´

spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Finally, the DNA was diluted to working concentration of 25 ng/µl.

Polymerase chain reaction and high resolution melt analyses

PCR and HRM analyses were done using the Rotor-Gene 6000 real-time rotary analyser (Corbett Research, Australia). The PCR amplifications were performed for each RAPD and SSR markers separately in 15 µl reaction volume. The reaction volume for RAPDs was made up of 1.5 µl of 25 ng/µl genomic DNA template, 1.5 µl of 5 *µ*M each of the RAPD markers, 4.5 µl of water, and 7.5 µl of KAPA Master Mix (2X) Universal (KAPA SYBR® FAST qPCR kit). Similarly, the same amounts of genomic DNA template and KAPA Master Mix together with 0.6 µl of 5 µM each of forward as well as reverse primers of SSR markers, and 4.8 µl of water were used for the PCR reactions. A negative control with no DNA template in the PCR reaction was included as one sample in running PCR and gel-electrophoresis for both RAPD and SSR markers while a positive control (human DNA) was used for RAPD markers to ensure non-contamination of PCR reagents. The KAPA Master Mix (2X) Universal is a ready-to-use cocktail containing all components except primers and templates, for the amplification and detection of DNA in stringent real-time quantitative PCR (qPCR) reaction conditions. It is supplied as a 2X Master Mix with integrated antibody-mediated hot start, SYBR® Green I fluorescent dye, dNTPs, $MgCl_2$, and stabilizers used for efficient DNA amplification by the PCR system.

The PCR amplification was also done using similar procedures for both marker systems with a two minute hold at 95°C as an initial denaturation step, followed by 40 cycles of 95°C for 10 s, 37°C for 20 s for RAPDs (and 60°C for 15 s for SSRs), and 72°C for 20 s. For HRM analysis, the ramp was set up from 70 to 90° for RAPDs (and 75 to 85° for SSRs) rising by 0.2° for RAPDs (and 0.1° for SSRs) each step, and wait for 90 s of pre-melt conditioning on first step and wait for 2 s for each step on wards, after which the HRM analysis was performed automatically. Subsequently, amplified PCR products were electrophoresed on a 2% (w/v) agarose gel in 1 × TBE buffer at 150 V for 1½ h. The electrophoregrams of DNA were visualized and photographed under UV light after being stained with ethidium bromide. A low molecular weight DNA ladder reagent (6X) [1X Gel Loading Dye, blue: 2.5% Ficoll-400, 11 mM EDTA, 3.3 mM Tris-HCl (pH 8.0 at 25°C), 0.017% SDS, and 0.015% bromophenol blue] was added to the reactions for running the gel-electrophoresis.

Statistical analyses

Since polymorphic banding patterns were obtained for all the three RAPD markers, statistical analyses were performed for only RAPDs. Accordingly, gel photographs were scored manually and repeated more than once to reduce errors in scoring. The bands were binary coded by 1 or 0 for their presence or absence for each line. Each band was considered as one *locus*. Genetic distances (GD) and similarity (GS) matrix between genotypes based on the scored marker data was computed using the Nei's (1978) procedure (Table 2). Cluster analysis was conducted with the unweighted pair group method based on arithmetic averages (UPGMA) (Sneath and Sokal, 1973) to generate a dendrogram using version 1.3 of the TFPGA software (Miller, 1997). The discriminating power of a *locus* was determined by the information content of each marker system calculated for each *locus* using the polymorphism information content (PIC) (Smith et al., 1997) Calculations for PIC were made using the following formula:

$$PIC = 1 - \sum f_i^2 ,$$

where f_i is the frequency of the i^{th} allele.

RESULTS AND DISCUSSION

HRM analyses

The graphs of the HRM analyses showed fairly similar melt patterns for both marker systems (Figures 1 to 3). The melting temperature peaks of the RAPD markers for different cycles of DNA polymerization process is shown in Figures 1 and 2, while Figure 3 shows the HRM data for SSRs. Since two samples per inbred line were analysed separately, similar or a comparable melt pattern should have been displayed within inbred samples as opposed to between inbred samples. However, deviations from this expectation were observed for the three figures of HRM analyses.

For inbred lines Q2, whose graph was represented by Q21 and Q22 with a pale green colour, and Q7 represented by Q71 and Q72 with faint yellow colour, a

Table 2. Nei's unbiased measures of genetic identity and genetic distance for the eight lines.

Line ID	1	2	3	4	5	6	7	8
1	****	0.9677	0.5484	0.7742	0.6452	0.4516	0.6129	0.7419
2	0.0328	****	0.5806	0.7419	0.6774	0.4839	0.6452	0.7742
3	0.6008	0.5436	****	0.5161	0.5161	0.5161	0.8710	0.7419
4	0.2559	0.2985	0.6614	****	0.7419	0.5484	0.4516	0.5806
5	0.4383	0.3895	0.6614	0.2985	****	0.7419	0.5806	0.7097
6	0.7949	0.7259	0.6614	0.6008	0.2985	****	0.5806	0.5806
7	0.4895	0.4383	0.1382	0.7949	0.5436	0.5436	****	0.8710
8	0.2985	0.2559	0.2985	0.5436	0.3429	0.5436	0.1382	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

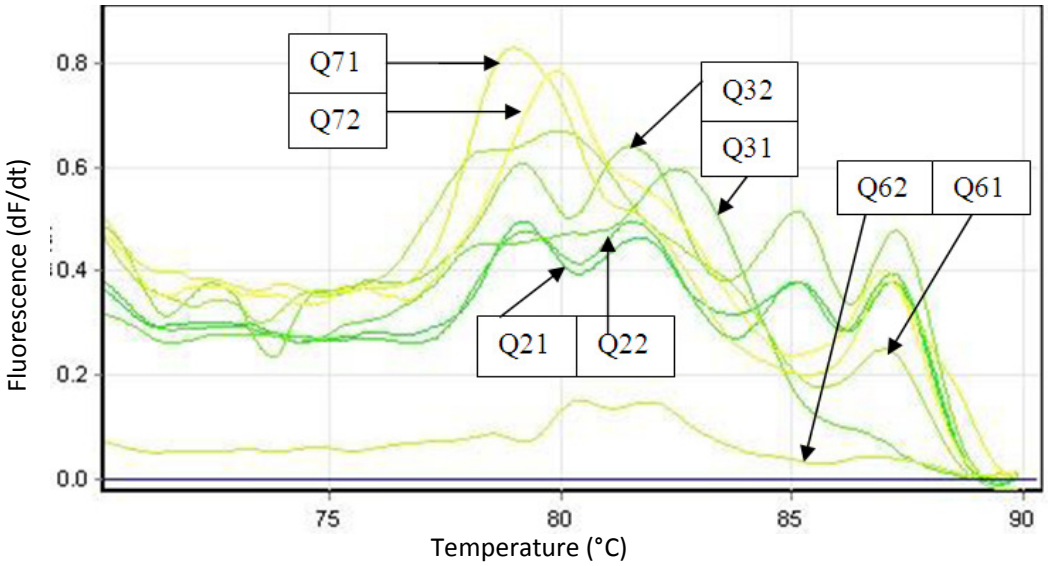

Figure 1. Melt curve for HRM analysis using RAPD (*OPA-12* as a primer).

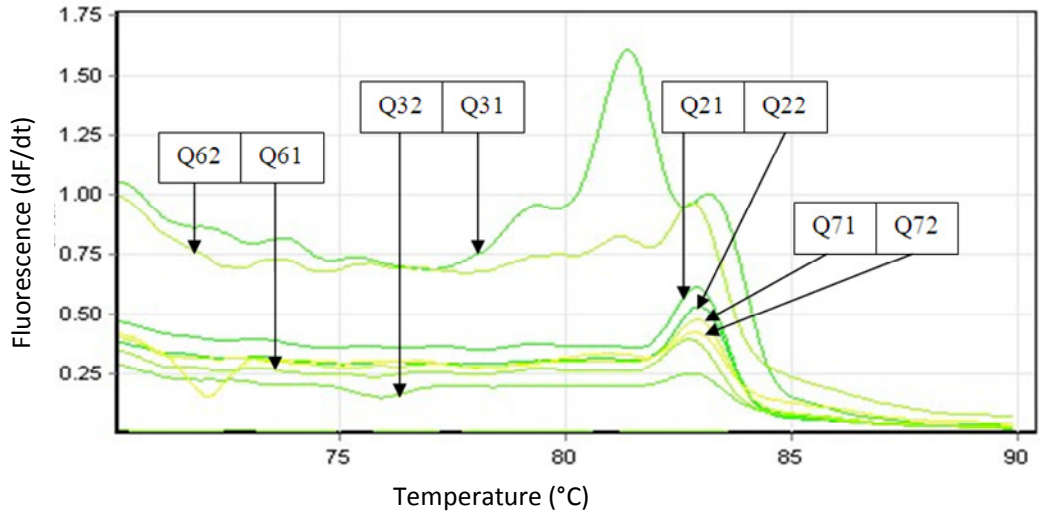

Figure 2. Melt curve for HRM analysis using RAPD (*OPA-16* as a primer).

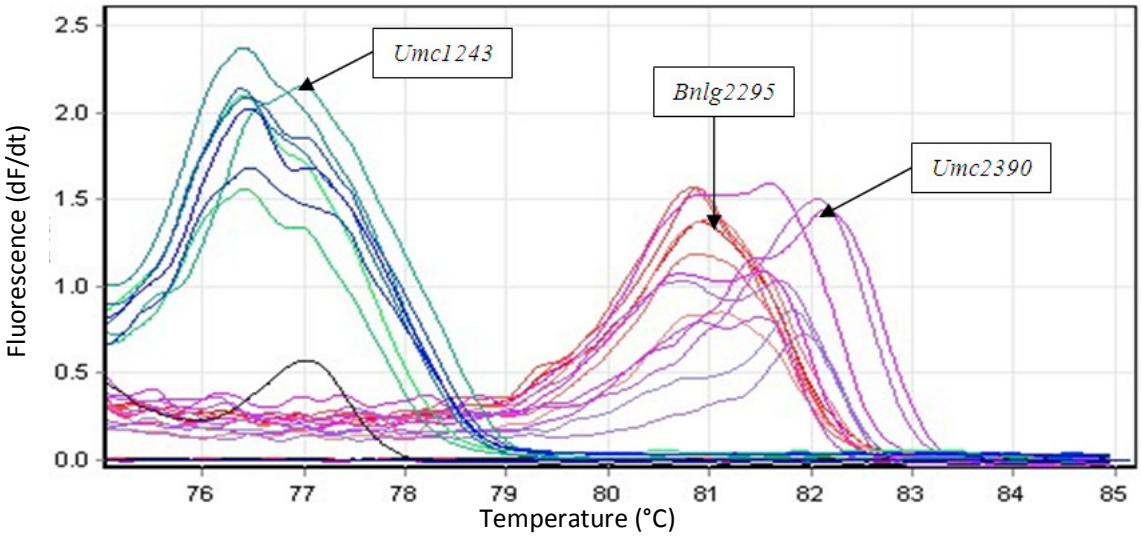

Figure 3. Melt curve for HRM analysis using three SSR primers.

relatively similar patterns of melt peaks were observed in the two samples collected from the same inbred line (Figure 1); whereas, the pattern observed for the other two inbred lines, Q3 and Q6, was different from the expected trend in that melt peaks' pattern for the two samples of the same inbred line (Q31-Q32, and Q61-Q62) had no resemblance suggesting that these lines, Q3 and Q6, were not pure inbreds, that is, pollen or seed stock contamination might have occurred in these inbred lines.

Although Figure 2 does not show clear differences as such among all the lines, clear variations in melt peaks were observed among some of the lines. For example, melt peak of Q3 and Q6 groups were totally different from that of Q2 and Q7 groups indicating that there was variation within and between the four genotypes at least for that amplified region of the locus. Similarly, HRM melt data were analysed for the third RAPD marker (OPA-18) in which too many and complex graphs or banding patterns were observed (data was not shown).

In Figure 3, the HRM analysis for the three SSR markers as shown by three separate groups of different coloured graphs with arrows is presented. It was only one of the SSR markers (Umc2390- purple coloured graphs) that showed apparent differentiation in melt peaks among and within the four inbreds following more or less similar trend as the RAPD marker, OPA-12, described earlier. Of the two remaining SSR markers, Umc1243 (green and blue coloured graphs), was monomorphic for that particular locus and hence incapable of discriminating the melt peaks or it was not possible to see the polymorphism at the given resolution. On the other hand, close analysis of the melt data for the third marker, Bnlg2295 (red coloured graphs), which did not clearly disclose variations in melt peaks among the lines, revealed that there were no amplification products for some of the inbred lines. In general, the HRM analyses carried out for both marker systems were able to show the existence of genetic variations among the DNA samples despite the relatively low quality DNA used in the present study.

Analyses of gel-electrophoresis

Results of the analysis of the gel electrophoresis of the present experiment were generally in agreement with the HRM analyses. For the RAPD markers, similar banding patterns were observed in the samples from inbred lines, Q2 (Q21 and Q22) and Q7 (Q71 and Q72) while the banding patterns of the samples obtained from Q3 (Q31 and Q32) and Q6 (Q61 and Q62) (Figure 4) were different, thus suggesting again that there might have been contamination either in the seed stocks or from external pollen as was pointed out earlier for the HRM analysis. These similarities and differences in the size of bands within inbreds might have arisen from the amplification of DNA fragments of different sizes by all the three RAPD primers. Furthermore, differences in banding patterns were not only detected within the two samples of the same inbred lines but also between the four inbred lines themselves. However, relatively clear polymorphic banding patterns were visualized in OPA-12 than the other two RAPD primers, OPA-16 and OPA-18 (Figure 4). On the other hand, the three SSR markers also showed different banding patterns on their own such that only marker Umc2390 was able to display clear polymorphic banding patterns, while monomorphic patterns were observed for marker Umc1243 suggesting that this primer might have amplified similar region of a locus common to all the inbred lines as all the inbreds

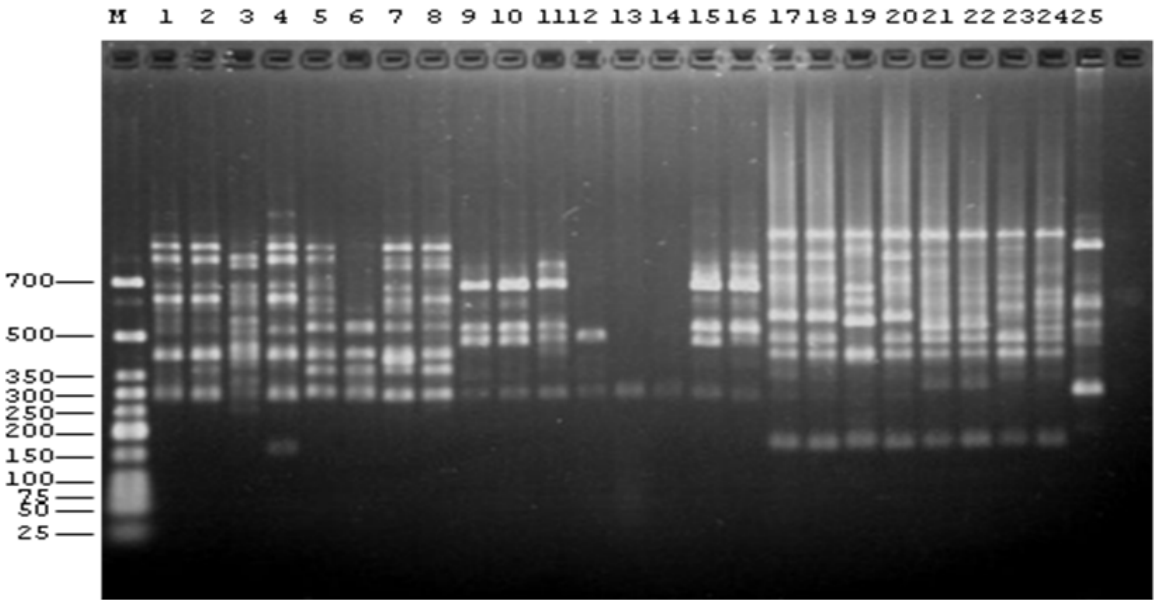

Figure 4. Agarose gel electrophoregrams of three RAPD primers (OPA-12, OPA-16, and OPA-18 from left to right); where: M = molecular weight ladder, 1 = Q21, 2 = Q22, 3 = Q31, 4 = Q32, 5 = Q61, 6 = Q62, 7 = Q71, 8 = Q72, and the same lines were replicated in that order from 9-16 and 17-24 across the remaining two markers. Number 25 represents the control (human DNA).

had the QPM trait and tropical adaptation. There was no amplification product observed from the third marker Bnlg2295 for two samples (Q22 and Q31) of inbreds Q2 and Q3, respectively, as mentioned earlier in the HRM analyses (Figure 5).

Comparison of the two marker systems in this experiment (Table 3) revealed that the RAPDs produced several polymorphic bands although the resolution power of the agarose gel-electrophoresis was not good enough to allow the bands of both marker systems to be seen clearly. A total of 31 alleles were detected for the 25 polymorphic RAPD loci, at an average of 1.24 alleles per locus which is also equivalent to 80.7% polymorphic loci. Thirty seven out of 40 RAPD primers showed monomorphic banding pattern while three RAPD primers exhibited polymorphic bands. These results are consistent with the finding of Asif et al. (2006). However, the PIC value was better for the SSR marker although the value calculated was only for a single marker. It is, therefore, interesting to note the discriminating power of SSR markers which makes them ideal for use in fingerprinting of maize lines as reported by Smith et al. (1997) and Liu et al. (2003). Garcia et al. (2004) also reported that the RFLP and SSR polymorphism information content (PIC) means were higher than the RAPD and AFLP means. Similar comparisons of different DNA markers used for diversity studies in maize have been made to evaluate the relative efficiencies of the different techniques (Smith et al., 1997; Pejic et al., 1998; Garcia et al., 2004). For example, the results of the study by Garcia et al. (2004) demonstrated that except for RAPD markers all the other three markers (SSRs, RFLP and AFLP) provided consistent information for diversity studies on tropical maize populations and produced relatively close estimates of genetic distances. Therefore, the use of more than one marker in the present study was advantageous as recommended earlier by Warburton et al. (2002) because using a single marker may not provide authentic information for reliable and accurate differentiation of maize genotypes.

The SSR profile in our study concurs with the results of Kassahun and Prasanna (2003) in that most of the inbred lines displayed deviation from the assumption that inbreds are supposed to be highly homozygous and, hence, are expected to reveal only a single amplification product (allele) per locus. However, the codominant nature of the SSR markers made possible the detection of 'double bands' which otherwise would have been hidden if only RAPD (dominant) markers had been used (Figure 5). The occurrence of such 'double-bands' or SSR heterozygosity is a good signal to justify that the lines were not homozygous at the specific locus. This is actually not uncommon for inbred lines developed entirely through inbreeding and successive phenotypic selections because of residual heterozygosity being left during the process. In other words, the SSR markers in the present study facilitated the differentiation of homozygotic and heterozygotic alleles in the inbred lines from the same origin. A study by Shehata et al. (2009) using SSRs for molecular diversity and heterozygosity analysis also showed that different seed sources of the same inbred were such an important source of genetic variations that

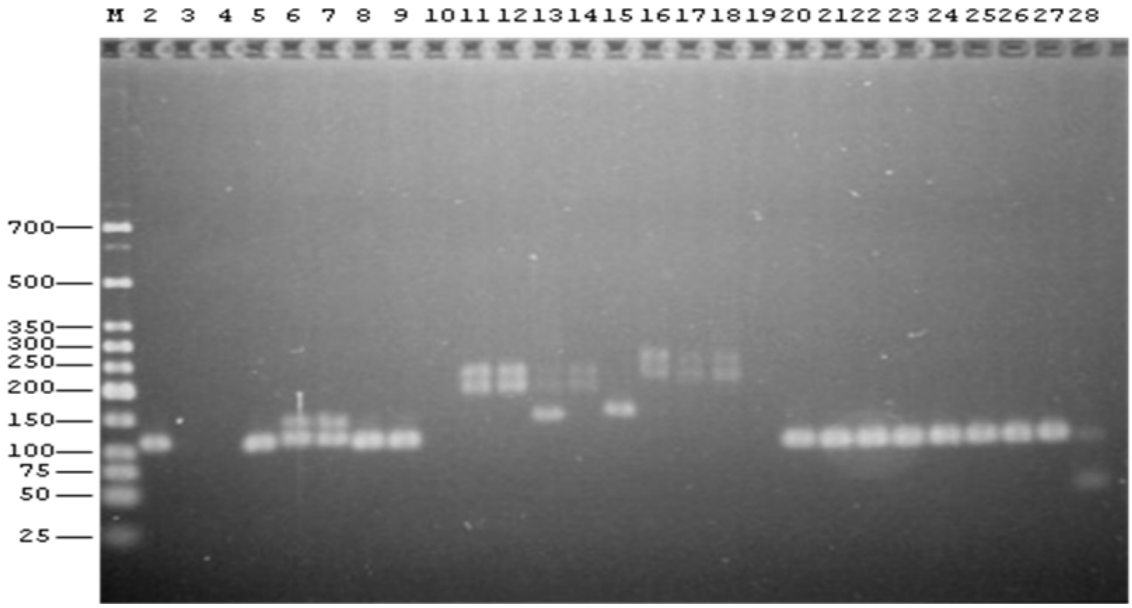

Figure 5. Agarose gel electrophoregrams of three SSR primers (Umc1234, Umc2390, *Bnlg2295*- from left to right); where: M = molecular weight ladder, 2 = Q21, 3 = Q22, 4 = Q31, 5 = Q32, 6 = Q61, 7 = Q62, 8 = Q72, and the same lines were replicated in that order from 11 to 18 and 20 to 27 across the remaining two markers. Numbers 10, 19 and 28 represent the no template control.

Table 3. Number of bands scored for four primers that showed clear polymorphism.

Marker/primer		Total number of bands scored as presence in all samples	No. of alleles	PIC*
RAPD	*OPA-12*	68	12	0.37
	OPA-16	34	6	0.25
	OPA-18	66	12	0.52
SSR	*Umc2390*	14	6	0.78

* = Polymorphic information content.

there is a need to be aware of the variation arising from seed sources if we are to use the inbreds continuously for genetic studies and as testers. Furthermore, low levels of variations within an inbred from a single source should not be overlooked, especially, during sampling of materials developed years ago. This is because a wide range of genotypic dynamism can occur in cross pollinating crops such as maize (Hallauer et al., 2010).

The dendrogram obtained from the UPMGA clustering algorithm based on genetic similarities calculated for RAPD markers showed that most of the inbred lines could be distinguished from each other (Figure 6), and were clustered into four groups. Clusters containing the most closely related inbred lines were cluster I and III. Again, it was noted that the cluster analysis also supported the deviations observed in the results of the HRM analyses and gel-electrophoresis such that Q31 and Q32 (represented as 3 and 4), and Q61 and Q62 (represented as 5 and 6) in the dendrogram should have been either in the same cluster or should have exhibited minimum genetic distances, respectively, as they were two samples from the same inbred.

Conclusion

In the present study, the two types of marker systems in combination with HRM analyses were able to detect genetic differences both within and between inbreds. Therefore, it will be advisable to adopt a rapid procedure for pre-screening of large number of samples of newly developed inbred lines using only HRM analysis so that running of gel-electrophoresis at early stages could possibly be omitted in order to save time and resources.

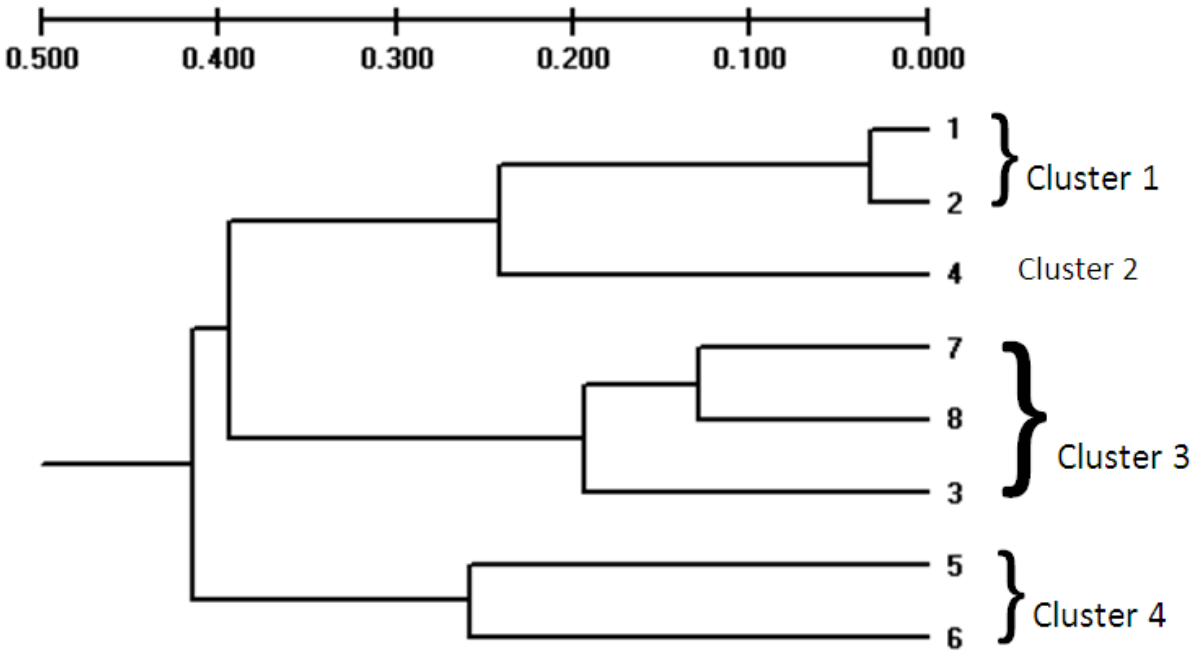

Figure 6. UPGMA clustering of eight inbreds using Nei (1978) unbiased minimum distance.

We also recommend that the reliability of results of experiments designed to detect genetic differences between inbreds could be improved by increasing the number of markers and the number of samples from each inbred.

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ABBREVIATIONS

HRM, High resolution melts; **QPM,** quality protein maize; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphisms; **SSR,** simple sequence repeats; **CTAB,** cetyltrimethylammonium bromide; **GD,** genetic distance.

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