

## EVALUATING GENETIC PURITY OF QUALITY PROTEIN MAIZE (QPM) INBREDS AND HYBRIDS BY BIOCHEMICAL AND SSR MARKERS

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### ABSTRACT

Four maize hybrids and their parental lines were used to find genetic purity through testing with four isoenzymes such as alcohol dehydrogenase (ADH), esterase (EST), acid phosphatase (ACP) and malate dehydrogenase (MDH) and evaluated with thirty pairs of simple sequence repeat (SSR) markers. Out of the four isoenzymes, only malate dehydrogenase could able to detect polymorphism between parental lines of the two hybrids and confirmed the hybridity. The enzyme was also able to distinguish the hybrids from their female parent. However, it was failed to detect the off types in the parental lines and in the hybrids. Out of 30 SSR markers employed for assessing genetic purity of hybrids, seven SSR markers were found polymorphic and confirmed the hybridity. Out of seven SSR markers, only one primer (dupssr34) distinguished the three-way cross hybrid Shaktiman-1 from its single cross female parent. Using this primer, 87.5% genetic purity was confirmed in the hybrid. These results clearly demonstrated that dupssr34 marker could be successfully used to evaluate genetic purity of three-ways cross QPM hybrid, Shaktiman-1, and may be used in seed production programme.

**Key words:** Genetic purity, *Isoenzyme*, QPM, SSR marker.

### INTRODUCTION

Maize is an important cereal food crop in the world after wheat and rice. It is a staple food for millions of people in poor countries around the world. Worldwide, maize is cultivated on an area of 165 million hectares with a total production of 1040.21 million metric tons giving an average yield of 3.19 metric tons/ha (Anonymous, 2015). Quality Protein Maize (QPM) possesses roughly twice as much usable protein for which it has widely been adopted for cultivation in the developing world to fight protein malnutrition. It was released in India for commercial cultivation almost a decade ago by introducing QPM lines from CIMMYT. Later on India has developed an expertise in this area and since then as many as eight single cross hybrids have already been released. The genuineness of the variety is one of the most important characteristics of good quality seed and an essential pre requisite for the commercialization of any hybrid seed. In hybrid maize technology, incomplete removal of tassel, cross pollination from adjacent field, mechanical mixtures while handling the seed during postharvest operations, etc. deteriorate the purity and quality of the seeds that consequently decreases the crop productivity. It is estimated that for every 1 per cent impurity in the hybrid seed, the yield reduction is 100 kg per hectare (Mao *et al.*, 1996). Therefore, one of the challenges is to assess and maintain purity of hybrid seeds so that they can be supplied to the farmers in time and at low cost.

Conventionally, grow out test (GOT) based on morphological traits is followed for certification of genetic purity of hybrid maize. However, this method is affected by environmental conditions making unreliable determination of identity (Yashitola *et al.*, 2002). Moreover, this test is time consuming and hybrid seed is not immediately available for cultivation as the seed production is carried out in succeeding season. Several high precision technologies such as biochemical and molecular markers are also suggested as alternative/additional methods for the purity identification of hybrids. Maize is one of the important crop where detailed isozyme analyses have been conducted and exhaustive information on the method of analyses, genetic control and molecular nature are available (Stuber and Goodman, 1983). Isozymes are becoming biochemical markers of choice for initiating or advancing genetic studies of plants (Karaca, 2013). Of the many isoenzymatic systems, malate dehydrogenase (MDH), acid phosphatase (ACP), phosphoglucose isomerase (PGI), 6-phosphogluconate dehydrogenase (PGD) and isocitrate dehydrogenase (IDH) were reported to be most informative for characterization of maize inbreds (Mauria *et al.*, 2000). Indeed, many isoenzymatic systems recommended by International Seed Testing Association (ISTA) have been commonly used in checking seed-lot purity in maize and has been dealt by many authors (Orman *et al.*, 1991; Smith and Wych, 1995; Salgado *et al.*, 2006, Nikolic *et al.*, 2008).

DNA based markers, particularly the co-dominant markers, such as Simple Sequence Repeat

(SSR) have been proved to be preferred molecular marker for purity identification in hybrid maize as well as other crops because they are highly abundant, dispersed throughout plant genome, showed co-dominant inheritance, easy to score (Varshney *et al.*, 2005; Xu *et al.*, 2013), are more informative because it can detect multiple alleles per locus (Xu *et al.*, 2013) and highly polymorphic even in closely related individuals (Gupta *et al.*, 2002).

In this study, four isozymes (esterase, alcohol dehydrogenase, malate dehydrogenase and acid phosphatase) and thirty SSR markers were employed for evaluating their application in purity identification of three-way cross QPM hybrid Shaktiman-1, single cross QPM hybrid Shaktiman-2 and their parental lines.

## MATERIALS AND METHODS

**Isozyme analysis:** In this study, polymorphism among parental lines of two F<sub>1</sub> hybrids Shaktiman-1 [(CML 142 × CML 150) × CML186] and Shaktiman-2 (CML 176 × CML186) with respect to the esterase (EST), alcohol dehydrogenase (ADH), malate dehydrogenase (MDH) and acid phosphatase (ACP) was analyzed. Pooled samples of 10 coleoptiles of each of the hybrids and their parental lines in two replications were used for extraction of crude enzymes except for ADH for which seed was used. After detecting the polymorphism and confirming the hybridity with the isoenzyme pattern, fifty individual coleoptiles were analyzed for detecting off types in the hybrids and parental lines. ADH isoenzyme was isolated from seed using 75mM sodium phosphate extraction buffer, pH 7.5 (Percy and Wendel, 1990). Rest three isoenzymes were isolated from coleoptile using extraction buffer (0.1M Tris HCl, pH 7.5) at 4°C. Isoenzymes were resolved on 8% alkaline native polyacrylamide gel system with 4% stacking gel (Davis, 1964) at 4°C using a Bio-Rad protean II electrophoresis unit running at constant current of 30 amps per gel with voltage set at maximum. The gels were stained for esterase (EST) as per the procedure of Glaszmann (1987), acid phosphatase (ACP) Vallejos (1983), alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) Shaw and Prasad (1970).

### Molecular analysis

**DNA extraction and amplification:** Leaf samples were collected in bulk and from individual plant of the hybrid and its parental lines with two replications. In case of the hybrid Shaktiman-1, leaf samples were also collected from forty individual plants. DNA was isolated from leaves of 15-days old seedlings following modified CTAB method of Saghai-Marooof *et al.* (1984). Extracted DNA was further purified using the phenol-chloroform method and quantified by analyzing DNA on 1% agarose gel using diluted uncut lambda DNA as standard.

DNA amplification was carried out with a thermal cycler (MJ PTC-100) in a 11µL reaction mixture containing 25ng DNA, 10X PCR buffer (Bangalore genei), 25mM MgCl<sub>2</sub> (Bangalore genei), 10mM dNTP mix (MBI fermentas), 10 µM each SSR primers, 3 U *Taq* polymerase (Bangalore genei). Thermo cycling consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturing at 94°C for 1 min, primer annealing (58-65°C) for 2 min and primer extension (72°C) for 2 min. The last cycle consisted of primer extension (72°C) for 4 min followed by termination and maintenance at 4°C. Amplified DNA fragments were separated on a 3.5 % SFR (Super fine resolution; Amresco) agarose gel in 0.5X TBE along with 50bp DNA ladder (Bangalore genei) at 100v for 2 hrs and stained with 10mg/ml ethidium bromide. The gel was visualized under UV and photographed.

The QPM genotypes used in this study were first identified with opaque 2 locus specific codominant simple sequence repeat (SSR) marker, phi 057 present within the opaque 2 loci. Thirty pairs of SSR primers/markers chosen from maize database (<http://www.maizegdb.org>) were used for genetic purity analysis in one hybrid (Shaktiman-1).

## RESULTS AND DISCUSSION

In this study, application of four biochemical marker systems (*viz.* EST, ADH, ACP and MDH) and thirty SSR markers were evaluated for assessing the genetic purity of two QPM hybrids, Shaktiman-1 (three way cross hybrid), Shaktiman-2 (single cross hybrid) and parental lines.

Electrophoresis analysis of isoenzymes is known to provide a reliable assessment in testing genetic purity of hybrids and inbred lines of maize (Orman *et al.*, 1991; Wang *et al.*, 1994). Among the four isoenzymes analyzed, three namely esterase, alcohol dehydrogenase and acid phosphatase showed a monomorphic electrophoresis profile among the hybrids and their respective parents (Fig.1) which is corroborated with the results of Salgado *et al.*, (2006). However, only malate dehydrogenase (MDH) exhibited good degree of polymorphism among inbreds and hybrids studied. Malate dehydrogenase was reported to be effective in identifying hybridity (Bilgen *et al.*, 1995) and also informative isozyme systems for characterization of inbred (Mauria *et al.*, 2000). A total of 10 bands were amplified in individual genotypes with 6 bands in CML 142 to 10 bands both in Shaktiman-1 and Shaktiman-2. Hybridity could be established in case of Shaktiman-1 and Shaktiman-2 (Fig. 2) by the presence of marker band(s) contributed by each of the parental lines (Table 1). Isoenzymes being co-dominant in nature could distinguish heterozygous F<sub>1</sub> hybrid from the homozygous

progenies. Three-way cross hybrid Shaktiman-1 was distinguishable from single cross female parent (CML142 × CML150) by the presence of a band having Rm value of 0.43. Similarly, single cross hybrid Shaktiman-2 was discriminated from female parent (CML176) by the presence of three bands having Rm value 0.31, 0.36 and 0.54. This result of differentiation of hybrid from their parents is in agreement with the results of Bilgen *et al.* (1995) and Nikolic *et al.* (2008) but contradicts results of Salgado *et al.* (2006).

Fifty single coleoptiles of both the hybrids and their parental lines were analyzed with this isozyme to detect off types. However, this isozyme could not able to detect any off types neither in the parental lines nor in the hybrids (Fig.3). It is reported that this effective method is sometimes limited by its failure in detecting polymorphism in some closely related lines (Wu *et al.*, 2006).

This suggested that MDH could not be efficiently used for purity testing of these two QPM hybrids. Even though isozyme analysis in maize has been recommended by ISTA (International seed testing association) for purity identification and is considered as useful characteristics and only be used as a complement to other differences in morphological or physiological characteristics but might not be sufficient on their own to establish distinctness (UPOV 2009). Therefore, molecular markers, based on variation in DNA-sequence can be applied for identifying crop varieties as they would be precisely assessing the genotype of a plant. Among the molecular markers, SSR/microsatellites markers are ideal and recently used technologies for assessing the genetic purity of hybrids due to its high efficiency and simplicity (Wu *et al.*, 2006).

Thirty SSR primers were used for screening polymorphism between parental lines of one QPM hybrid, Shaktiman-1. Pooled DNA sample of 15 seedlings (two replications) of parents and hybrid were used for detecting polymorphism and confirming hybridity. Seven out of thirty primers (viz. bnlg 1065, bnlg 2323, dupssr 23, bnlg 589, bnlg 1209, bnlg 1452 and dupssr 34) showed polymorphism between parents (Fig. 4). Remaining twenty-three markers were found to be monomorphic among the parental lines and hybrid studied. These seven polymorphic primers confirm the hybridity by amplifying two alleles both in the single cross female parent (CML 142×CML 150) and in hybrid Shaktiman-1 where as amplified single band in the male line (CML 186) except at dupssr34 locus.

The dupssr34 marker resulted in heterozygous profile in the three way cross hybrid Shaktiman-1 by amplifying two alleles in the size range of 115 bp and 100 bp in male parent (CML 186) and female parent (CML 142 × CML 150), respectively. The single cross female parent (CML 142 × CML 150) was homozygous at this locus so the heterozygous status of hybrid

Shaktiman-1 was easily identifiable and distinguishable from female parent (Fig. 4g). This result suggested that the dupssr34 marker having both female and male specific bands is useful for identifying three ways cross QPM hybrid, Shaktiman-1. However, this marker could have been used to identify other QPM hybrids because if other QPM hybrids would have similar SSR profiles as that of Shaktiman-1 then this marker may fail to establish the identity of the hybrid seed lot.

The effectiveness of the identified marker in ensuring the genetic purity of hybrid depends on homogeneity/uniformity in the individual seedling/plant constituting the hybrid. Hence, homogeneity of the identified polymorphic SSR marker of the particular hybrid was confirmed in 40 individual seedlings of the hybrid. Out of 40 individual seedlings analyzed with dupssr34 marker, 35 seedlings were of hybrid Shaktiman-1 type (Fig. 5). Thus, genetic purity of the three-way cross hybrid Shaktiman-1 was amount to 87.5% which suggested that it can be suitably used for assessing genetic purity of Shaktiman-1. However, an additional marker could have been used to ascertain seed purity of Shaktiman-1 for validation of this result depending on the consideration of the cost for conducting the assay. It need to be discussed here that the purity/homogeneity of parental lines of this hybrid could have been assayed by using this marker so that purity of hybrid determination is not impaired due to negative determination of off types/selfed plants. Small changes in allele frequencies may occur during regeneration, maintenance at two different places, bulking during maintenance breeding and possible contamination with seeds or pollen of other samples (Heckenberger *et al.*, 2002; Warburton *et al.*, 2010). Significant changes in the genetic makeup of a germplasm may affect performance and in the worst case result in distribution of wrong hybrids or varieties (Semagn *et al.*, 2012).

This QPM hybrid seed collected from Kholapur, Maharashtra, India was found contaminated with selfed seed (12.5%) which may have arisen either through self pollination of female parent or mechanical mixtures. Hybrid maize is produced by crossing two pure lines with high combining ability. Since maize is a monoecious crop, tassel from female line is removed prior to anthesis in order to avoid selfing. Incomplete removal of tassel from the female parent results in genetic contamination of hybrid seed with the self seed. Also maize being a highly cross pollinated crop, there exists always chances of contamination under traditional maize growing areas and seasons. This contamination reduces the genetic and physiological quality of hybrid seeds that consequently decreases the crop productivity. This suggests that for maintaining the purity and superior quality, complete removal of tassel from the female parent should be ensured while producing QPM hybrid seed and further spatial and temporal isolation should be ensured either

through growing in non-traditional areas or seasons so as to avoid cross pollination with normal maize which may dilute the QPM trait. In India, a total of eight QPM hybrids, of which 7 single cross hybrids, 1 three-way cross hybrid having different maturity period and grain colour, have been developed by the public sector Institutes. These cultivars have been developed and found suitable for their cultivation under different agro-climatic conditions having compatibility under different cropping systems and preference of the local people.

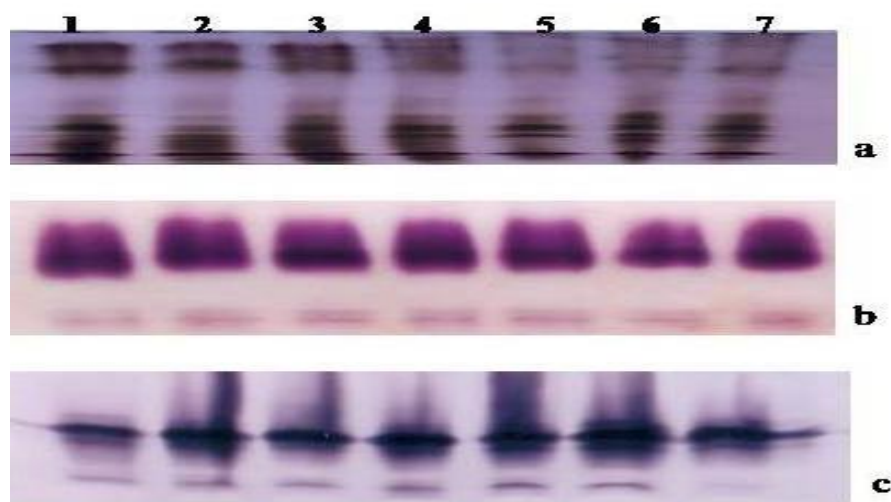
Monitoring the seed quality particularly the genetic purity of inbreds as well as hybrids is required for commercial success of hybrid maize technology. In maize, mostly GOT is carried out for assaying genetic purity. Locking up of the capital invested on hybrid seed production, additional expenditure incurred on storage of hybrid seed for a whole season, cost of acquiring land and growing the crop for the GOT, ultimately increases the cost of hybrid seed. In recent years, with the availability of more than 2000 pairs of SSR primer publicly in the Maize Data base

(<http://www.maizegdb.org/ssr.php>), with one SSR marker per every 15.48 kb (Xu *et al.*, 2013) has greatly improved their utility. Also the high polymorphic content (PIC) of SSR markers had promoted its application in fingerprinting (Ashikawa *et al.*, 1999). Though high initial cost involved and lack of expertise has reduced its application to a certain extent but it can be overcome by optimizing the sample size and by developing the expertise without compromising the reliability of the test. Use of such markers will not only simplify the identification of hybrid but also substantially reduce the time, space, labor and ultimately the cost involved in testing genetic purity of hybrid seed through morphological characteristics. Environmental dependence and the limitation imposed by GOT can be managed effectively by SSR markers and it would be a good option for establishment of an effective seed quality control system in commercial seed production of QPM hybrids keeping in view the gaining importance of QPM hybrids in tribal belt of Assam, North East Hilly region, Bihar and in Southern part of India.

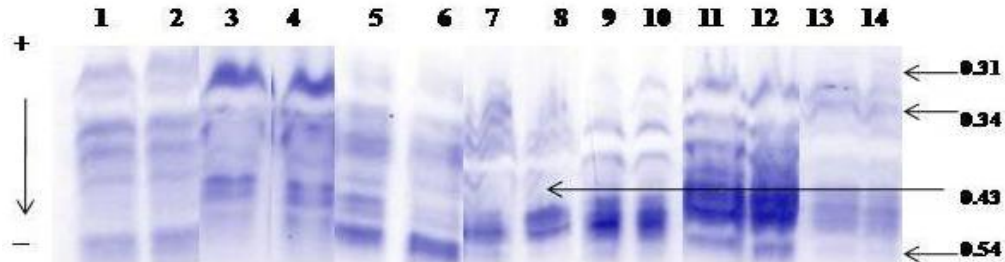
**Table 1. Malate dehydrogenase (MDH) isoenzyme profiles of hybrid Shaktiman-1, Shaktiman-2 and their parents\*.**

Genotype	Rm value of bands													Total bands
	0.31	0.33	0.34	0.36	0.38	0.41	0.43	0.45	0.47	0.48	0.49	0.51	0.54	
CML 142	1	0	1	0	1	1	0	1	0	0	0	1	0	6
CML 150	1	0	1	1	1	1	0	1	1	0	1	0	0	8
CML142 X CML 150 (F)	1	0	1	1	1	1	0	1	1	0	1	1	0	9
CML 186 (M)	1	0	0	1	1	0	1	0	0	0	1	1	1	7
Shaktiman-1 (H)	1	0	1	1	1	1	1	1	1	0	1	1	0	10
CML 176(F)	0	1	0	0	1	1	1	1	0	0	1	1	0	7
Shaktiman-2 (H)	1	1	0	1	1	1	1	1	0	0	1	1	1	10

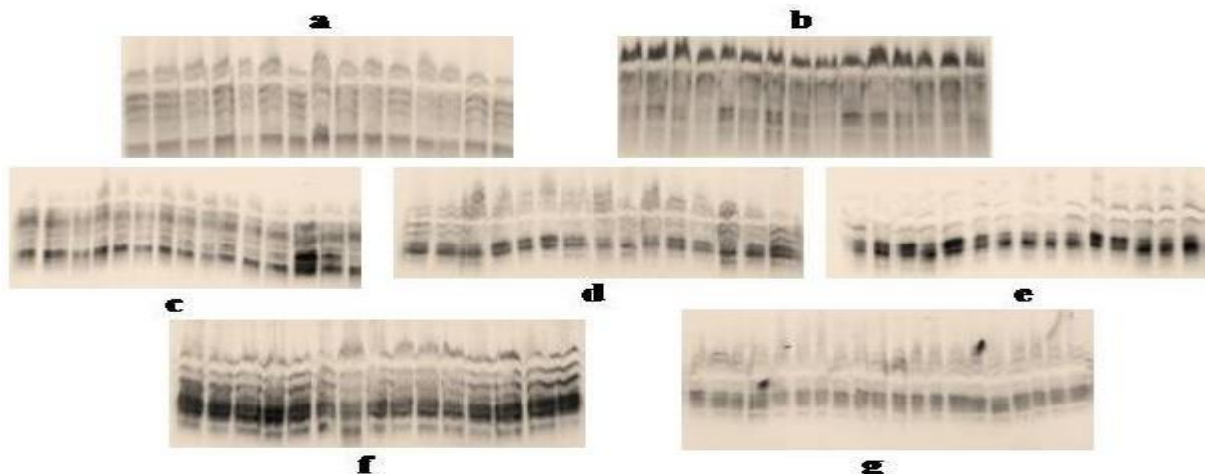
\* F - Female parent; M - Male parent; H - Hybrid ; 1-presence of band;0-absence of band



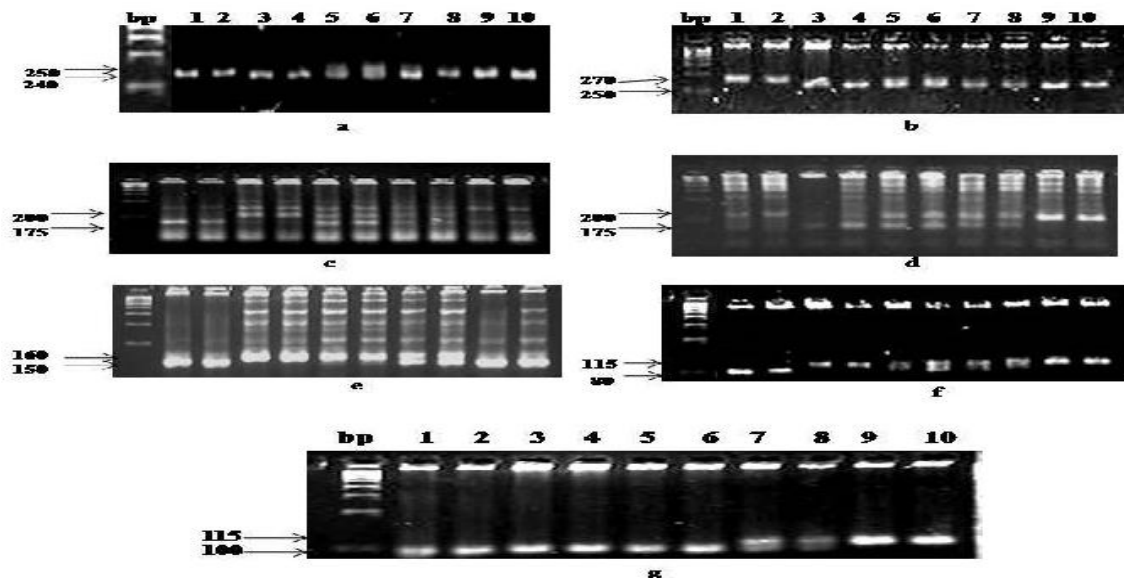
**Figure 1. Monomorphic isoenzyme profile in QPM hybrids and parental lines with esterase (a), acid phosphatase (b), alcohol dehydrogenase (c) . Lane 1-CML142, Lane 2- CML150, Lane 3- CML142x CML150, Lane 4- Shaktiman-1, Lane 5-CML186, Lane 6- CML -176, Lane 7-Shaktiman-2**



**Figure 2.** Malate dehydrogenase isoenzyme profiles in QPM hybrids and parental lines. Lane 1 & 2 -CML142 RI & RII, Lane 3 & 4 CML 150 RI & RII, Lane 5 & 6 -CML142xCML150 RI & RII, Lane 7 & 8 Shaktiman-1 RI & RII, Lane 9 & 10-CML186 RI & RII, Lane 11 & 12-CML176 RI & RII, Lane 13 & 14-Shaktiman-2 RI & RII

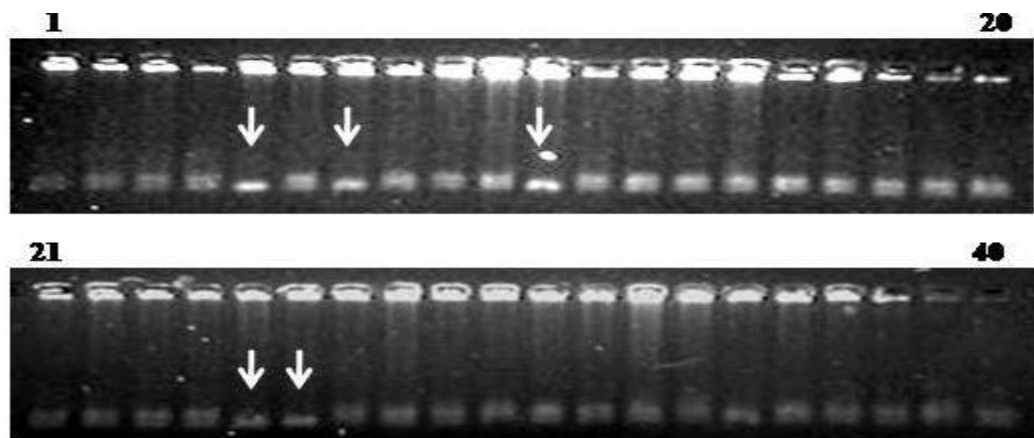


**Figure 3.** Malate dehydrogenase isoenzyme profiles in single seedlings of parental lines and hybrids of Shaktiman-1 and Shaktiman-2 (a) CML142, (b) CML150, (c) CML142xCML150, (d) Shaktiman-1 (e) CML186, (f) CML176, (g) Shaktiman-2



**Figure 4.** SSR marker polymorphism between parental lines and three-way cross QPM hybrid Shaktiman-1 obtained with seven polymorphic SSR marker\*.

\*(a) bnlg 1065, (b) bnlg2323, (c) dupssr 23, (d) bnlg1452, (e) bnlg 589, (f) bnlg 1209, (g) dupssr34. Lane 1 & 2 - CML142 RI & RII, Lane 3 & 4 - CML 150 RI & RII, Lane 5 & 6-CML 142XCML150 RI & RII, Lane 7 & 8- Shaktiman-1 RI & RII, Lane 9 & 10-CML 186 RI & RII.



**Figure 5.** SSR profiles in single seedlings of hybrid Shaktiman-1 using primer dupssr34. Off types are indicated by arrows present in lanes 5, 7, 11, 25 & 26.

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