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Assessment of Hybrid Purity in Maize (*Zea mays* L.) Using RAPD and SSR Markers

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Abstract
Maize (*Zea mays* L.; 2n=20) is third most important cereal crop and referred as the "Queen of Cereals" and "Miracle Crop" due to its high productivity potential as compared to the other cereals. Maize is native to Mexico and Central America. Maize is the most important crops in the world that can be grown in diverse seasons, ecologies it is used mainly for human food, animal feed and industry. This work was carried out to assess the hybrid purity of maize hybrid Hyd.18227 × Hyd.10306 (Tall) and their parental line Hyd.18227 (Dwarf) and Hyd.10306 (Tall) using RAPD and SSR markers. DNA extracted from young leaves and PCR were conducted using 24 RAPD and 18 SSR markers. Out of fifteen RAPD markers and five SSR makers showed the polymorphism between the maize parental lines and remaining primer produced monomorphic banding pattern. The SSR primer Umc-1858 identified 64 % hybrid seeds of total cross.

Keywords: Maize, F_1 hybrids, hybrid purity, RAPD, SSR

1. Introduction
Maize is third most important cereal crop after rice and wheat in the world. Maize is referred as the "Queen of Cereals" and "Miracle Crop" due to its high productivity potential as compared to the other cereals. Maize is native to South America. Maize is diploid with chromosome number 2n=20. Maize (*Zea mays* L.) is popularly known as corn. The important maize growing countries are USA, China, Brazil, Mexico, India, Philippines, South Africa and Indonesia. In India, maize has been grown in an area of 9.2 million ha with production of 24.17 MT and average productivity of 2.56 t/ha (USDA 2017-18). In Maharashtra the area under maize is increasing every year. The area under maize in Maharashtra is about 2 million ha with a production of 3.45 MT and productivity of 3.24 t/ha.

Conventionally, purity of F_1 hybrids is assessed by grow-out test (GOT) at the field. This test is time consuming and resource intensive (Elci and Hancer, 2014) [8]. To overcome this disadvantage, biochemical markers are being used in many crops. However, repeatability and accuracy of these results on biochemical markers are subject to question. This made a way for the use of DNA markers particularly the co-dominant markers. Hence, it is essential to develop a more rapid, accurate and cost effective method for the identification of maize hybrids. Considering the disadvantages of grow out test, rapid and reliable methods using biochemical and molecular markers are getting attention for genetic purity testing. Now several biochemical and molecular markers *i.e.*, RAPD, ISSR, AFLP, SSR etc. are being used.

Molecular marker technology provides effective, fast, accurate and appropriate tool for crop improvement. DNA markers such as RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeats), CAPS (Cleaved Amplified Polymorphic Sequences), RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism), SNPs (Single Nucleotide Polymorphisms), Sequence characterised amplified regions (SCAR), Expressed sequence tag (EST) and sequence tagged sites (STS) have been used for varietal identification, seed purity testing, genetic similarity analysis and marker-assisted selection of crops in many species. SSRs, also known as microsatellites, are repeated sequences of DNA and they can easily detect both parental alleles, confirmation of F_1 hybrid purity and diversity in maize hybrids by using molecular markers are more accurate because of their co-dominancy. Also, SSR marker efficiency was analyzed for further studies on maize.

The objective of this study was assessment of hybrid purity in F_1 populations.

2. Materials and Methods

Seeds of maize hybrid namely Hyd.18227 × Hyd.10306 (Tall) and parental line Hyd.18227 (Dwarf) and Hyd.10306 (Tall) was collected from Department of Botany. The experiment was conducted at Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur (M.S.) during the year 2017-18.

2.1 DNA extraction and Quantification

DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) protocol given by Doyle and Doyle (1990) with some modifications and quantification was done by Spectrophotometer. DNA was diluted in 0.1 T.E buffer to a concentration of 50 ng/micro liter for PCR analysis.

2.2 Primer Screening and polymerase chain reaction

Twenty four random RAPD primers (GeNei) were used for the present investigation. The list of the primers with their sequences is given in the below in Table No. 1. PCR reaction were performed using a 25 µl reaction mixture containing 10X PCR buffer with $MgCl_2$, 10 mM dNTPs, 10 pmol primer, 1 U of *Taq* DNA polymerase, 50 ng/µl template DNA and sterile distilled water. For DNA amplification the DNA thermal cycler (Sensoquest Labcycler, Germany) was programmed as follows: incubation at 94°C for 5 min; 35 cycles at 94°C for 30 sec (denaturation), 37°C for 30 sec (annealing) and 72°C for 30 sec (extension), followed by one final extension cycle of 10 min at 72°C. After completion of the cycles the samples were kept at 4^0C till electrophoresis.

Eighteen SSR primers (Eurofins Genomics, India) were used for the present investigation. The list of the primers with their sequences is given in the below in Table No. 2. PCR reaction were performed using a 20 µl reaction mixture containing 10X PCR buffer with $MgCl_2$, 10 mM dNTPs, 10 pmol of each primer (both forward and reverse), 1U of *Taq* DNA polymerase, 50 ng/µl template DNA and sterile distilled water. For DNA amplification the DNA thermal cycler (Sensoquest Labcycler, Germany) was programmed as follows: incubation at 94°C for 10 min; 40 cycles at 94°C for 30 sec (denaturation), 58°C for 50 sec (annealing) and 72°C for 1:30 min (extension), followed by one final extension cycle of 10 min at 72°C. The annealing temperature was adjusted based on the specific requirement of each primer combination.

The amplified products were resolved on 2 % agarose gel for SSR markers, 1.5% for RAPD marker at 5V/cm for 1 to 1.5 hr. After electrophoresis, the gel was taken out for observation of banding pattern and photographed on a Gel Documentation System (Alpha-Innotech, USA).

2.4 Genetic Purity Test of Hybrid Seeds

The percentage of hybrid genetic purity were calculated based on banding pattern that appears on the individual plant samples, with the following formula:

$$\text{Purity hybrid (\%)} = \left\{1 - \left[\frac{NH}{TS}\right]\right\} \times 100\%$$

Where: TS (total sample) = number of samples/individual plants were tested

NH (non-hybrid) = number of samples/individual plants having the same banding pattern with female or male parents.

3. Results and Discussion

3.1 Development of F_1

In present investigation, two parental maize genotypes were used for crossing of which one genotype was dwarf and remaining genotype was tall. The Hyd.18227 (Dwarf) used as female and Hyd.10306 (Tall) used as male parent. These genotypes were crossed successfully to obtained tall hybrid. The F_1 hybrid seeds were harvested from particular cross after seed set and maturity.

3.2 DNA isolation and quality analysis

The genomic DNA was extracted from young leaves of maize hybrid and their respective parents by Cetyl Trimethyl Ammonium Bromide (CTAB) DNA extraction method given by Doyle and Doyle (1990) [7] with some modifications. This method yielded qualitatively as well as quantitatively pure genomic DNA. The quantification of extracted DNA was done by measuring absorbance at 260 nm wavelengths. Purity of DNA was checked by reading absorbance ratio of A260/280 for protein contamination. The quantitative and qualitative analysis was done by resolving DNA on 0.8% agarose gel. The concentrations of all samples were ranged between 500-1400 ng/µl. Working samples were prepared by diluting with sterile nuclease free water to obtain final concentrations of 50 ng/µl for RAPD and SSR analysis.

3.3 Hybrid confirmation based on RAPD fingerprint profile analysis

RAPD analysis would be very useful in breeding for rapid and early verification of hybrid population and even purity testing of different seed lots, allowing the detection of true hybrids and verification of parentage of the hybrids and lines/cultivars. RAPD analysis has been successfully used for hybrid and parentage verification of other crop plants. RAPD marker fingerprinting data was used for hybrid confirmation. The present study utilized total 24 RAPD markers for identification of maize hybrid (Hyd.18227×Hyd.10302) along with their female parental line (Hyd.18227) and male parental line (Hyd.10302). The hybrid confirmation discussed here under was done by comparing banding patterns of hybrids with their respective parents as described by Akhare *et al.* (2008) [1]. Different types of markers have been designated for hybrid confirmation as per convenience in Coffea arabica (Mishra *et al.*, 2011) and cotton (Mehetre *et al.*, 2004) [11, 10]. For hybrid purity analysis, the total of six types of banding patterns was observed in the parents and hybrids (Table No. 3).

Table No 1: List of RAPD primers used for hybrid purity assessment

Sr. No.	Primer ID	Sequence	Sr. No.	Primer ID	Sequence
1	RPI 1	5'AAAGCTGCGG3'	13	RPI 13	5'ACGGCAAGGA3'
2	RPI 2	5'AACGCGTCGG3'	14	RPI 14	5'ACTTCGCCAC3'
3	RPI 3	5'AAGCGACCTG3'	15	RPI 15	5'AGCCTGAGCC3'
4	RPI 4	5'AATCGCGCTG3'	16	RPI 16	5'AGGCGGCAAG3'
5	RPI 5	5'AATCGGGCTG3'	17	RPI 18	5'AGGCTGTGTC3'

6	RPI 6	5'ACACACGCTG3'	18	RPI 19	5'AGGTGACCGT3'
7	RPI 7	5'ACATCGCCCA3'	19	RPI 20	5'AGTCCGCCTC3'
8	RPI 8	5'ACCACCCACC3'	20	RPI 21	5'CACGAACCTC3'
9	RPI 9	5'ACCGCCTATG3'	21	RPI 22	5'CATAGAGCGG3'
10	RPI 10	5'ACGATGAGCG3'	22	RPI 23	5'CCAGCAGCTA3'
11	RPI 11	5'ACGGAAGTGG3'	23	RPI 24	5'CCAGCCGAAC3'
12	RPI 12	5'ACGGCAACCT3'	24	RPI 25	5'GAGCGCCTTC3'

Table No 2: List of SSR primers used for hybrid purity assessment

S. No.	Primer ID	Forward	Reverse
1	Phi053	CTGCCTCTCAGATTCAGAGATTGAC	AACCCAACGTACTCCGGCAG
2	Phi057	CTCATCAGTGCCGTCGTCCAT	CAGTCGCAAGAAACCGTTGCC
3	Phi080	CACCCGATGCAACTTGCGTAGA	TCGTCACGTTCCACGACATCAC
4	Phi96100	AGGAGGACCCCAACTCCTG	TTGCACGAGCCATCGTAT
5	Phi328175	GGGAAGTGCTCCTTGCAG	CGGTAGGTGAACGCGGTA
6	Phi034	TAGCGACAGGATGGCCTCTTCT	GGGGAGCACGCCTTCGTTCT
7	Phi299852	GATGTGGGTGCTACGAGCC	AGATCTCGGAGCTCGGCTA
8	pumc1064	GTGGGTTTTGTCTGTAGGGTGGTA	TCCATCCACTCGACTTAAGAGTCC
9	pumc1013	TAATGTGTCCATACGGTGGTGG	AGCTGGCTAGTCTCAGGCACTC
10	pumc1746	ACACGAGCATCCTACATCCTCCTA	ACCTTGCCTGTCCTTCTTTCTCTT
11	pumc1071	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT
12	pumc1040	CATTCACTCTCTTGCCAACTTGA	AGTAAGAGTGGGATATTCTGGGAGTT
13	pumc1035	CTGGCATGATCACGCTATGTATG	TAACATCAGCAGGTTTGCTCATTC
14	pumc1066	ATGGAGCACGTCATCTCAATGG	AGCAGCAGCAACGTCTATGACACT
15	Umc-1858	GTTGTTCTCCTTGCTGACCAGTTT	ATCAGCAAATTAAAGCAAAGGCAG
16	Umc-1600	CATATTGATAGGCTAGGCAAATGGC	CAATACAAGTTTGGTCCCAAATAAGC
17	Umc1605	CCAGGAGAGAAATCAACAAAGCAT	GGAGAAGCACGCCTTCGTATAG
18	Umc1331	TTATGAACGTGGTCGTGACTATGG	ATATCTGTCCCTCTCCCACCATC

Table No 3: Types of RAPD markers observed in hybrid and their parents

Markers Type	Female (F)	Male (M)	Cross (H)	Nature	Remark
1	+	+	+	Monomorphic	Good marker to confirm hybrid of its respective parents (male and/or female
2	-	+	+	MPS	
3	+	-	+	FPS	
4	+	-	-	-	Good markers to identify self and off types
5	-	+	-	-	
6	-	-	+	Cross specific	Useful for identification of cross

+ indicates presence of band while – indicates absence of band.
MPS-Male parent specific band
FPS-Female parent specific band

Out of twenty four RAPD primers fifteen primers were found to be polymorphic and nine primers were found to be monomorphic. Confirmation of hybrid was achieved by six primers amplified the specific allele size of RPI-1 (370 bp), RPI-8 (900 bp), RPI-10 (1200 bp), RPI-18 (400 bp), RPI-15 (700-500 bp), and RPI-20 (390 bp) in hybrid and male parent but not amplified in female parent. These six primers were used to confirm the true hybrid. These primers produced MPS band of Type 2 maker (Fig. No 1). The two primers RPI-2 and RPI-25 amplified the specific allele of size 1200 bp and 280 bp respectively in hybrid and female parent but not amplified in male parent. These primers produced FPS band of Type 3 maker and used for hybrid confirmation (Fig. No.2). Three primer *viz.*, RPI-5, RPI-6 and RPI-7 amplified the specific allele size (1100 bp), (600 bp) and (1000 bp) respectively in male parent but not amplified in hybrid and female parent. These primers produced Type 5 maker and good markers to identify off types (Fig. No.3). Two primers *viz.*, RPI-4 and RPI-19 produced female parent specific band~1200 bp and~ 600 bp respectively. These primers produced Type 4 marker and good markers to identify self and off types (Fig. No.3). RPI-24 and RPI-21 produced the cross specific band size 250 bp and 300 bp respectively. These primers produced Type 6 marker and useful for identification of specific cross (Fig. No.3).

Similar investigations based on RAPD analysis have been successfully employed for parentage verification, hybrid confirmation, cultivar identification and purity testing in maize (Asif *et al*, 2006; Mrutu 2015) [2, 12] and other crops such as sorghum (Akhare *et al*., 2008), rice (Deshmukh *et al*., 2013) [1, 5] and cotton (Asif *et al*, 2009 and Dongre *et al*. 2005) [3, 6].

3.5 Hybrid confirmation based on SSR fingerprint profile analysis

From eighteen primers tested five primers were found polymorphic and thirteen primers were found monomorphic. Primer Umc-1858 produced polymorphic bands and was capable to distinguish parental line of maize hybrid. Microsatellite marker Umc-1858 was specific used for testing genetic purity of Hybrid (Hyd.18227×Hyd.10306). The SSR primer Umc-1858 had amplified allele of size 170 bp in female parent (Hyd.18227).On the other hand the male parent (Hyd.10306) had an amplicon at 180 bp. However, hybrid (Hyd.18227×Hyd.10306) exhibited the alleles of both parents confirming the heterozygosity of the hybrid by having two bands at 170 and 180 bp. These primers produced both MPS Type 2 maker and FPS Type 3 marker (Fig.No.4). Thus, it

confirmed that the presence of both female and male parent alleles was observed as a resultant of crossing between two parents (F_1 hybrid). The Phi-328175 and Pumc-1746 primers amplified a specific allele size of 270 bp and 130 bp respectively in cross and female parent but not in male parent. These primers produced female specific band of Type 3 marker (Fig. No.4). The primer Pumc-1013 produced the female parent specific allele size 170 bp and male parent specific allele size 130 bp but not amplified in cross. These primer produced Type 4 and 5 markers (Fig. No.4). The primer Pumc-1066 amplified allele size 190 bp and 170 bp in cross but allele size 190 bp not amplified in female and male parents. These primer produced Type 6 marker and these band is useful for identification of specific cross (Fig. No.4).

The banding pattern of all these hybrids showed both the amplicons present in female as well as male parent, thus confirming the genuine crossing and heterozygotic condition of the hybrid. The use of SSR markers for genetic purity testing has already been demonstrated in maize (Daniel *et al.*, 2012; Mrutu, 2015; Wu *et al.*, 2010; Sudharani *et al.*, 2013 [4, 12, 14, 13]. In all the above studies SSR markers were used for germplasm identification, cultivar fingerprinting, true hybrid identification, genetic purity testing. Parentage confirmation of hybrids and identification of heterotic pattern in hybrids both female and male specific bands and are useful in genetic purity testing.

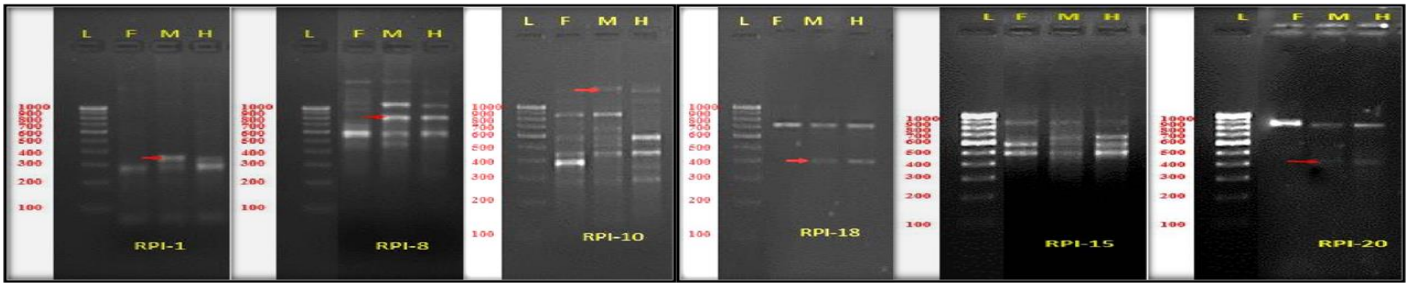

Fig No 1: Banding Profile of hybrid and parents obtained by Primer RPI-1, RPI-8, RPI-10, RPI-18, RPI-15 and RPI-20

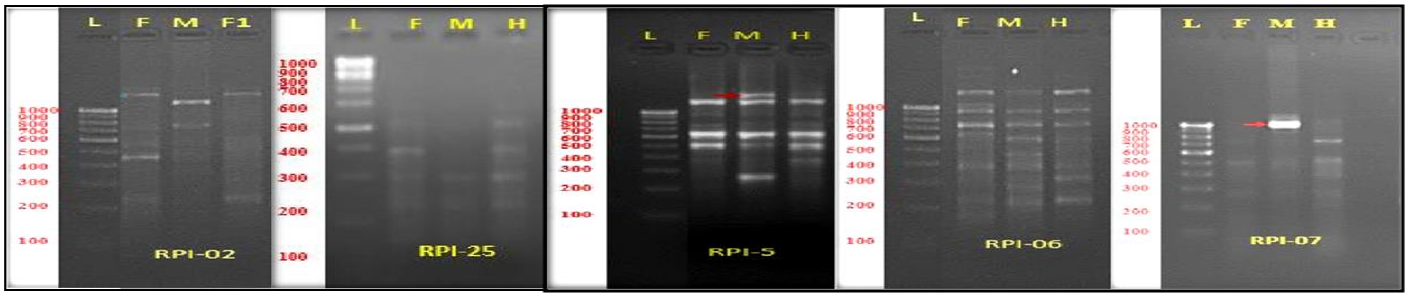

Fig No 2: Banding Profile of hybrid and parents obtained by Primer RPI-02, RPI-25, RPI-05, RPI-06 and RPI-07

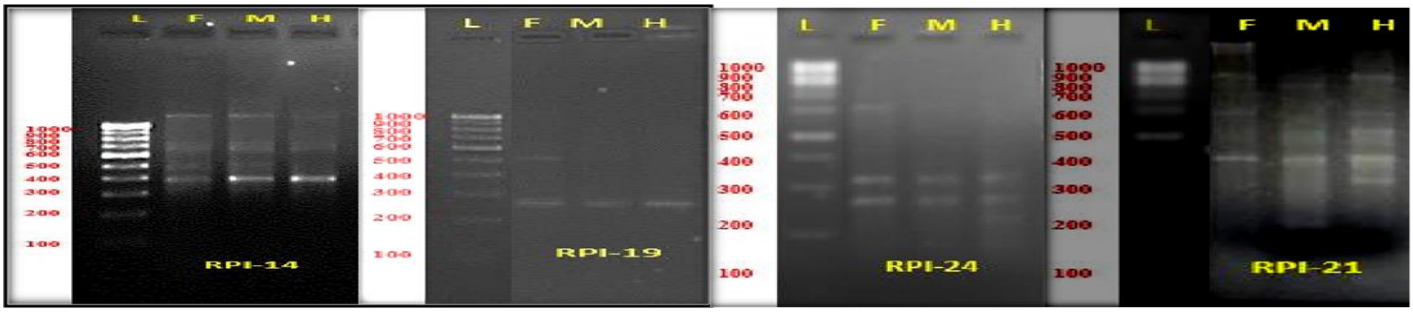

Fig No 4: Banding Profile of hybrid and parents obtained by Primer RPI-14, RPI-19 RPI-24 and RPI-21

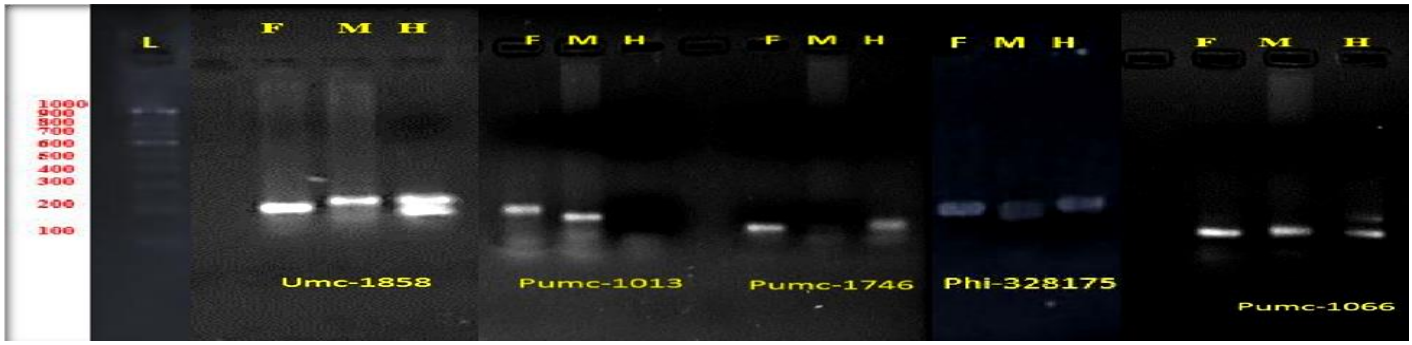

Fig No. 4: Banding Profile of hybrid and parents obtained by Umc-1858, Pumc1013, Pumc-1746, Phi-328175 and Pumc-1066

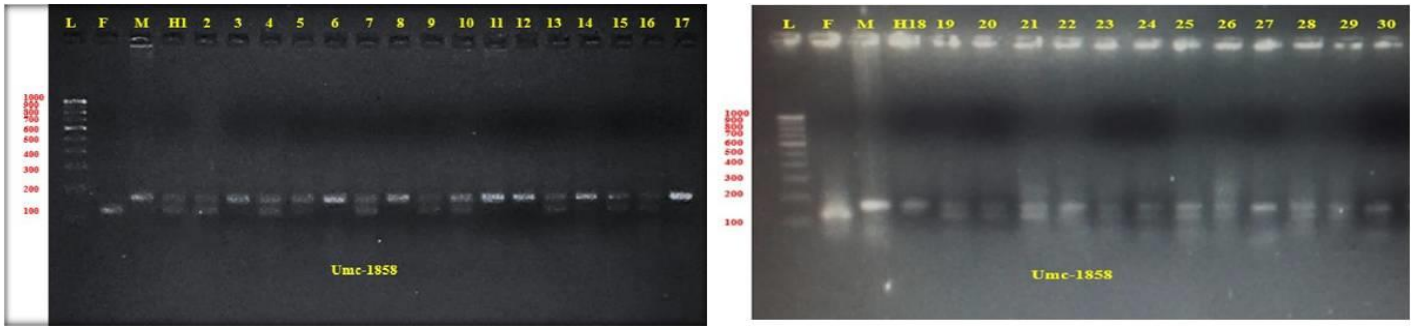

Fig 5: Banding profile of hybrid and parents obtained by Umc-1858

3.6 Genetic Purity Test of Hybrids

Thirty leaf samples of F_1 plants were taken for genetic purity testing with SSR markers. The percentage of hybrid genetic purity was calculated based on banding pattern that appears on the individual plant samples.

All 30 samples of maize cross were screened by using Umc-1858 of which 11 samples resembles male type band (Type 2 maker) and remaining sample produced male and female specific bands (Type 2 and Type 3 makers). Overall the total sample contained 64 % of the maize seeds that were genetically pure and 36 % of the maize seeds that were genetically impure (Fig. No.5).

The genetic purity test of maize hybrid was demonstrated by Hipi *et al*., (2013) [9] on selected 40 hybrid samples of *cv. Bima-4* identified using phi96100 marker, showed that seven samples which similar to the male parent bands, and one sample which similar female parent band. Overall the total sample contained 20% of the *cv. Bima-4* seeds that were not genetically pure.

Thus, the present study concluded that the PCR based molecular markers are likely to be promising for identification, registration and protection of commercial sample and will gain more and more influence on plant breeding in future and will speedup breeding processes considerably. The primer Umc-1858 screened with 30 maize hybrid samples contained 64% of the maize seeds that were genetically pure and 36% of the maize seeds that were genetically impure.

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