

DNA Aided Introgression of Opaque-2 Allele for Development of Quality Protein Maize

Rajesh Singh^{1*}, Lekha Ram¹

¹Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-Uttar Pradesh- 221005, India

*Corresponding Author E-mail- rsingh6361@gmail.com

Abstract: To improve the nutritional quality of maize, introgression of the opaque-2 (*o2*) trait to normal maize lines is essential. The QPM donors used in present study were; CML 161, DMR QPM 58, CML 176 and CML 141 whereas, normal maize inbreds were CM 212, V338, V361, V336, V341, V351, CM141 and V335. All the selected lines were subjected to parental polymorphism survey between non QPM and QPM lines using CIMMYT based three SSR primers viz., *phi057*, *umc1066* and *phi112*. Two markers viz., *phi 057* and *umc1066* exhibited co-dominant reaction while *phi 112* was dominant in nature. Finally two combinations CM141 × CML 176 and V335 × CML 161 were considered for conversion programme. Foreground selection was exercised using opaque-2 specific marker *umc1066* in BC₁ and BC₂ generations while background as well as foreground selection was exercised in BC₂F₁ generation to recover the genome of recurrent parent up to extent of 82.5 to 98.5 percent with the help of 98 SSR markers distributed across the genome. The tryptophan concentration in endosperm protein was significantly enhanced in all the three classes of kernel modification viz., less than 25%, 25-50% and more than 50% opaqueness. So, the converted maize lines had almost twice the amount of Lysine and Tryptophan than normal maize inbreds.

Keywords: QPM, Lysine, Tryptophan, Protein, Opaque-2

1. Introduction

The Quality Protein Maize (QPM) has about twice the levels of lysine and tryptophan as compared to normal maize. It was developed by combining the genetic systems of the gene mutant *opaque-2* (*o2*) and modified *o2* endosperm (Sofi et al., 2009; Krivanek et al., 2007; Babu et al., 2005; Prasanna et al., 2001 and Vasal, 2001). Although conventional breeding procedures to convert commercial lines to QPM forms, are tedious and time consuming and is not directly related towards improvement of grain quality. Rapid advances in genome research and molecular technology have led to the use of DNA marker assisted selection, which holds promise in enhancing selection efficiency and expediting the development of new cultivars with higher yield potential. The CIMMYT has confirmed that three SSR primers viz., *phi 057*, *phi 112* and *umc 1066*, which are located as internal repetitive elements within *opaque-2* gene. These three primers can be used to select individuals carrying a copy of the *opaque-2* gene in successive backcrossed and selfed segregating generations. In the present study we have studied to combine protein quality and hard endosperm traits through a combination of marker aided and phenotypic selection techniques. The studies also establishes that foreground selection for *opaque-2* in early (BC₁) generation combined with background selection for recipient genome at later (BC₂) generation results in rapid genetic gain and substantial cost savings.

2. Materials and Methods

Numbers of QPM and Non QPM lines were evaluated to screen inbreds lines which may be useful for molecular marker assisted breeding for Quality Protein Maize. Foreground selection for *opaque-2* was performed using two SSR primers viz., *phi057*, and *umc1066* located as

internal repetitive elements within *opaque-2* gene. For the frequent selection *phi057* or *umc1066* were used to identify heterozygous progenies carrying *opaque-2* gene in backcross generation, whereas marker *phi112* exhibited dominant polymorphism between normal and QPM inbreds. Based on parental polymorphism studies finally two Non QPM line; CM 141 and V 335 and two QPM line CML 176 and CML 161 were selected for conversion programme.

Genomic DNA was isolated from leaf samples of 21-25 days old seedlings of all the genotypes. DNA was utilized for parental polymorphism survey and marker assisted selection. For genomic DNA isolation, CTAB method (Saghai-Marooof et al., 1984) was used with some modification. PCR cycling consisted of initial denaturation at 94°C for 2 min, followed by 30-35 cycles of amplification at 94°C for 1 min, 55-65 for 1 min, and 72°C for 2 min. A final extraction step at 72°C for 7 min was followed by termination of the cycle at 40°C. The amplified products (15 ul) were resolved in a 3.5.1 SFR (Amresco) agarose gel at 125 V according to Senior et al. (1998). The gels were photographed and saved with the help of Gel documentation System GeNei™ for further analysis. Among the studied inbreds, the F₁ were attempted using recurrent parents V335 and CM 141 as female and QPM Donor CML 161 and CML 176, respectively, as male parent during winter 2011-12. F₁s of V335 × CML 161 and CM141 × CML 176 were backcrossed with respective recurrent parents (V335 and CM141) to obtain BC₁ generations. Two fold selection strategy (Babu et al., 2005) was adopted for selection in BC₁ generations. (A) Selection of heterozygotes for *opaque-2* gene specific to SSR marker *umc1066* (Fig. 3) (B) the selected heterozygotes were subjected to phenotypic selection and the genotype that resembled close to the Recurrent Parent (RP). The selected BC₁

individuals were grown to raise the BC₂F₁. The selected progenies were selfed to produce BC₂F₂ seeds, the selected BC₂F₂ individuals were raised to obtain BC₂F₃ generations. About 183 SSR markers earlier identified for polymorphism studies of maize inbreds were used to screen the respective recurrent parents and donor parents to recover the Recurrent Parent Genome (RPG). These polymorphic markers were used in backcross generations to regain the Recurrent Parent Genome (RPG) in the respective cross. The 105 and 89 SSR primers were identified to be used in recovery of Recurrent Parent Genome (RPG) for the crosses V335 × CML 161 and CM141 × CML 176, respectively (Table 3).

The biochemical analyses for total protein content in the endosperm and tryptophan concentration in endosperm protein in each class of kernel modification were carried out according to standard procedures developed by Villegas (1975) and Villegas et al. (1984). In short, the grain samples were de-germed after removing the pericarp and finely ground. The resulting flour was defatted and total nitrogen content was determined by Microjeldahl procedure and percentage of protein was calculated by multiplying the N content with a factor of 6.25. The tryptophan concentration in endosperm protein was estimated by the colorimetric method of Hernandez and Bates (1969).

3. Results and Discussion

3.1 Parental Polymorphism Survey

The normal maize protein is of poor nutritional quality due to a deficiency in two amino acids (lysine and tryptophan) and high leucine-isoleucine ratio. A breakthrough came in the 1960s, with the discovery of the enhanced nutritional quality of the maize mutant opaque-2 (Mertz et al., 1964). The *opaque-2* gene is recessive and modifiers are polygenic. Their introgression into elite inbreds is not a straight forward procedure. Keeping this in view a rapid maize inbred conversion programme based on two generation back cross conversion programme with the help of foreground and background were followed by phenotypic selection for kernel modification and other desirable agronomic traits in subsequent selfed generations. The present investigation establishes successful conversion of normal maize inbreds to QPM version possessing high lysine and tryptophan content in kernels through marker assisted back crossing.

Earlier Babu et al. (2005) and Gupta et al. (2013) have also reported similar conversion using 3 SSR molecular markers to local maize inbred lines CM 212 and CM 145. Although 3 SSR polymorphic marker (*phi112*, *phi057* and *umc1066*) were available for *opaque-2* locus, but only *umc1066* showed polymorphism between recurrent parent and donor parent, which were inherited co-dominantly. In the present study polymorphism could be observed between the normal and QPM inbreds lines with all the 3 SSR markers (Fig-2). However, the nature of polymorphism was different with respect to *phi112*, which exhibited dominant (presence-absence) polymorphism, restricting its potentiality in identifying the three forms of

genotypes for the *opaque-2* gene. Nevertheless, such presence absence polymorphism is only of limited use because it could not be used in discriminating homozygous (QQ) and heterozygous (Qq) back cross progenies. With the help of these information two parental combinations were selected viz., V335 (Non QPM) vs CML 161 (QPM Donor) and CM141 (Non QPM) vs CML 176 (QPM Donor) and backcross progenies of crosses V 335 × CML 161 and CM141 × CML 176 were subjected to marker assisted and phenotypic selection. Foreground selection using *phi057* or *umc1066* could identify heterozygous (Qq) progenies that occurred about 50 % frequency in a given back cross population. The expected frequency of heterozygotes of *opaque-2* gene confirmed the protocol in this particular back cross population. Identification of heterozygotes in seedling stage prior to pollination aided in the rejection of non target BC progenies (dominant homozygous) resulting in substantial savings of labour and material resources. Only these marker identified heterozygotes were further advanced to 2nd backcross generation.

3.2. Marker Assisted Back Cross Breeding

Identification of heterozygotes in the seedling stages prior to pollination aided in the rejection of non target BC progenies resulting in substantial saving of labour and material resources. Gupta et al. (2013) and Babu et al. (2005) reported conversion of normal maize inbred lines into QPM version using SSR marker *umc1066* as co-dominant marker for screening parental lines as well as segregating backcross generations (BC₁ and BC₂). In the present study, the 8 normal maize inbreds and 4 QPM inbreds exhibited polymorphism between QPM and non QPM inbreds using SSR markers *umc1066*.

With the help of SSR primers *umc1066* foreground selection was exercised in BC₂ generation (Fig. 3), which helped in elimination of progenies having dominant homozygous (QQ) and retaining heterozygous (Qq) individuals having QPM gene for the crosses V335 × CML 161 and CM141 × CML 176, respectively. The foreground selection helped in elimination of about 50 % of undesirable progenies and retaining rest which had *opaque-2* genes. The marker assisted selected heterozygotes in BC₂ were subjected to background selection using 105 and 89 SSR markers, in the cross V 335 × CML 161 and CM141 × CML 176, respectively (Table-1). These SSR markers spanning over all the 10 chromosome of maize genome were effective for background selection to recover RPG. The recipient progenies genome content varied from 75.0 to 91.7% and 70.0 to 95.0%, respectively in two crosses (V335 × CML 161 and CM141 × CML 176) in analyzed BC₂ generations. Ears from BC₂ progenies in each cross that contained maximum amount of recurrent parent genome (RPG) were selected for rising the BC₂F₂ generation. The selfing of BC₂F₁ advanced the generation to BC₂F₂ and for this backcrossed segregating generation about 270 plants were maintained in each cross which were subjected to molecular screening which led to screening of 107 and 101 heterozygotes (Qq), which were retained and 60 & 81 dominant homozygotes (QQ) were rejected, further 72 and

64 recessive homozygotes (qq) were also retained in two crosses (V335 × CML 161 and CM141 × CML 176), respectively.

3.3: Evaluation and Development of New QPM Lines

The background selection was exercised with the help of 105 and 89 SSR markers in the respective crosses distributed across the all 10 linkage groups of the maize. The main aim of the background selection is to rapidly recover maximum proportion of recurrent parent genome at non target loci with the help of molecular markers distributed evenly throughout the genome (Young and Tanksley, 1988; Hospital et al., 1992; Visscher et al., 1996; Frisch et al., 1999). Previously similar studies of Frisch et al. (1999), Babu et al. (2005) and Gupta et al. (2013) have indicated that application of background selection in one later generation along with the foreground selection in each BC generations is very effective. It may be mentioned here that employing background selection in each BC generations may not be affordable by many research groups. In the present investigation the backcross programmes were subjected to background screening using 105 and 89 SSR markers in two crosses V335 × CML 161 and CM141 × CML 176, respectively. So, in the present study, a two generation marker based breeding programme was applied in both the generation and background selection was applied in BC₂ at non target loci. Babu et al. (2005) and Gupta et al. (2013) have also adopted similar breeding strategy by selecting first three BC₂ individual with highest proportion of recurrent parent genome for developing further BC₂F₂ families. As indicated by Babu et al. (2005), we employed foreground selection in an early (BC₁) generation combined with background selection at later stages (BC₂) along with the phenotypic selection for quantitative traits. BC₂F₁ was selfed to obtain BC₂F₂, where kernels segregated for hardness at different levels of modification. In each cross, it was observed through light box screening that 5 classes of kernel modification consisted viz. 0%, less than 25 %, 25 -50%, 50-75% and more than 75% opaque. Lopes and Larkins (1995) revealed existence of two additive modifier genes that significantly affect the endosperm modification in their population. Vasal et al., 1993 reported that *opaque-2* allele is recessive and the endosperm modifiers are polygenic with, no reliable molecular marker identified for kernel modification. Phenotypic screening of the individual kernel under transmitted light and selection of kernels that have less than 25% opaqueness is by far the most convenient and efficient strategy employed in the QPM breeding programme. The biochemical analysis of kernel modification showed that tryptophan concentration in protein is the chief indicator of protein quality which was enhanced between 83 to 117% in two crosses as compared to recurrent parents. Lysine proportion in protein was not estimated due to the strong positive correlation between to amino acid in endosperm protein (Pixley and Bjarnasan, 1993). There was improvement (1.02 – 1.5%) in protein content of converted lines (Table 2).

References

- [1] Babu, R., Nair, S.K., Kumar, A., Venkatesh, S., Sekhar, J.C., Singh, N.N., Srinivasan, G. and Gupta, H.S. 2005. Two generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). *Theor. Appl. Genet.* 111: 888–897.
- [2] Frisch, M., Bohn, M. and Melchinger, A.E. 1999. Comparison of selection strategies for marker assisted backcrossing of a gene. *Crop Sci.*, 39: 1295–1301.
- [3] Gupta, H.S., Babu, R., Agrawal, P.K., Mahajan, V., Hossain, F. and Thirunavukkarasu, N. 2013. Accelerated development of quality protein maize hybrid through marker-assisted introgression of opaque-2 allele. *Plant Breeding*, 132: 77–82.
- [4] Hospital, F., Chevalet, C. and Mulsant, P. 1992. Using markers in gene introgression programs. *Genetics*, 132: 1199–1210.
- [5] Lopes, M.A. and Larkins, B.A. 1995. Genetic analysis of opaque2 modifier gene activity in maize endosperm. *Theor. Appl. Genet.*, 119: 274–281.
- [6] Moro, G.L., Habben, J.E., Hamaker, B.R. and Larkins, B.A. 1996. Characterization of the variability in lysine content for normal and opaque2 maize endosperm. *Crop Sci.*, 36: 1651–1659.
- [7] Pixley, K.V. and Bjarnasan, M.S. 1993. Combining ability for yield and protein quality among modified endosperm opaque2 tropical maize inbreds. *Crop Sci.*, 33: 1229–1234.
- [8] Ribaut, J.M. and Hoisington, D.A. 1998. Marker assisted selection: new tools and strategies. *Trends in Pl. Sci.*, 3: 236–239.
- [9] Saghai-Marouf, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal locations, and population dynamics. *Proc. Natl. Acad. Sci. USA*, 81: 8014–8018.
- [10] Senior, M.C., Murphy, J.P., Goodman, M.M. and Stuber, C.W. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Sci.*, 38: 1088 –1098.
- [11] Sofi, P.A., Wani, S.A., Rather, A.G. and Wani, S.H. 2009. Quality protein maize (QPM): genetic manipulation for the nutritional fortification of maize. *J. Plant Breed. Crop Sci.*, 1: 244–253.
- [12] Tanksley, S.D. 1993. Mapping polygenes. *Annu. Rev. Genet.*, 27: 205–233.
- [13] Vasal, S.K., Srinivasan, G., Pandey, S., Gonzalez, C.F., Crossa, J. and Beck, D.L. 1993. Heterosis and combining ability of CIMMYT's quality protein maize germplasm I: lowland tropical. *Crop Sci.*, 33: 46–51.
- [14] Villegas, E. 1975. An integral system for chemical screening of quality protein maize In: High quality protein maize. Proc CIMMYT- Purdue Int Symp on protein quality in maize, El Batan, Mexico.
- [15] Villegas, E., Ortega, E. and Bauer, R. 1984. Chemical methods used at CIMMYT for determining protein quality in corn. CIMMYT, Mexico.
- [16] Visscher, P.M., Haley, C.S. and Thompson, R. 1996. Marker assisted introgression in backcross breeding programs. *Genetics*, 144: 1923–1932.

[17] Young, N.D. and Tanksley, S.D. 1989. RFLP analysis of the size of chromosomal segments retained around Tm-2 locus of tomato during backcross breeding. *Theor. Appl. Genet.*, 77: 353-359

Table 1: Polymorphic simple sequence repeat (SSR) markers used in the BC₂F₁ families and the recovery of the recurrent parent genome (RPG) in the best lines

| Chromosome (Ch)-wise RPG recovery in BC ₂ F ₁ | Ch 1 | Ch 2 | Ch 3 | Ch 4 | Ch 5 | Ch 6 | Ch 7 | Ch 8 | Ch 9 | Ch 10 | Total |
|---|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| V335 × CML 161 | | | | | | | | | | | |
| Total SSR loci | 17 | 12 | 13 | 16 | 7 | 8 | 6 | 5 | 12 | 9 | 105 |
| Maximum loci recovered | 15 | 11 | 10 | 12 | 6 | 7 | 5 | 4 | 11 | 8 | 89 |
| Maximum recovery (%) | 88.2 | 91.7 | 76.9 | 75.0 | 85.7 | 87.5 | 83.3 | 80.0 | 91.7 | 88.9 | 84.8 |
| CM141 × CML 176 | | | | | | | | | | | |
| Total SSR loci | 20 | 9 | 10 | 11 | 7 | 5 | 8 | 4 | 8 | 7 | 89 |
| Maximum loci recovered | 19 | 7 | 7 | 10 | 6 | 4 | 7 | 3 | 7 | 6 | 76 |
| Maximum recovery (%) | 95.0 | 77.8 | 70.0 | 90.9 | 85.7 | 80.0 | 87.5 | 75.0 | 87.5 | 85.7 | 85.4 |

Table 2: Agronomic and biochemical features of the recurrent parent (V 335), Donor Parent (CML 141) and converted QPM version of V 335 (BC₂F₂ line)

| Traits / Descriptors | Recurrent Parent | | QPM Donor Parent | Converted QPM version (BC ₂ F ₂ line) | |
|--|------------------|-------------|----------------------------|---|-----------------|
| | V 335 | CM141 | CML 176 | (HUQPM 3) V 335 | (HUQPM 4) CM141 |
| Plant Height (cm) | 132 ± 9.8 | 124.4 ± 5.5 | 129 ± 13.1 128 ± 13.5 | 133 ± 16.5 | 123.3 ± 7.2 |
| Ear Height (cm) | 56 ± 6.8 | 51.6 ± 7.0 | 51 ± 8.5 50 ± 7.9 | 56 ± 11.3 | 51.2 ± 8.4 |
| Days to 50 % Tasseling | 99.8 ± 2.5 | 93.6 ± 2.8 | 109.4 ± 3.5 111.5 ± 6.7 | 98.5 ± 4.1 | 93.5 ± 3.1 |
| Days to 50 % Silking | 103.7 ± 3.1 | 95.7 ± 2.7 | 116.3 ± 4.5 117.4 ± 4.5 | 103.7 ± 3.5 | 95.2 ± 3.2 |
| Days to 75 % Brown Husk | 141.9 ± 7.5 | 135.1 ± 5.4 | 151.7 ± 7.6 154.4 ± 8.5 | 140.5 ± 7.9 | 134.1 ± 5.4 |
| Reaction to Maydis blight (1-5) ^a | 2.49 ± 0.18 | 2.90 ± 0.21 | 2.10 ± 10 2.17 ± 13 | 2.15 ± 0.20 | 1.90 ± 0.10 |
| Reaction to Turcicum Blight | 2.41 ± 0.18 | 3.10 ± 0.20 | 2.51 ± 17 2.54 ± 18 | 2.17 ± 0.21 | 2.00 ± 0.11 |
| Total Protein Content (%) | 8.47 ± 0.12 | 8.10 ± 0.13 | 8.70 ± 0.16 8.72 ± 0.18 | 8.66 ± 0.17 | 8.30 ± 0.21 |
| Tryptophan in Protein (%) | 0.42 ± 0.06 | 0.35 ± 0.07 | 0.81 ± 0.12 0.82 ± 0.13 | 0.77 ± 0.02 | 0.76 ± 0.10 |
| Kernel Hardness | Hard | Hard | Hard | Hard | Hard |
| Grain Yield ^b (g./plant) | 55.01 ± 3.04 | 44.2 ± 2.03 | 37 ± 3.7 38 ± 3.5 | 54 ± 3.7 | 43 ± 2.03 |

Mean ± Standard error

a 1-Resistant and 5-Susceptible

b Upon Selfing

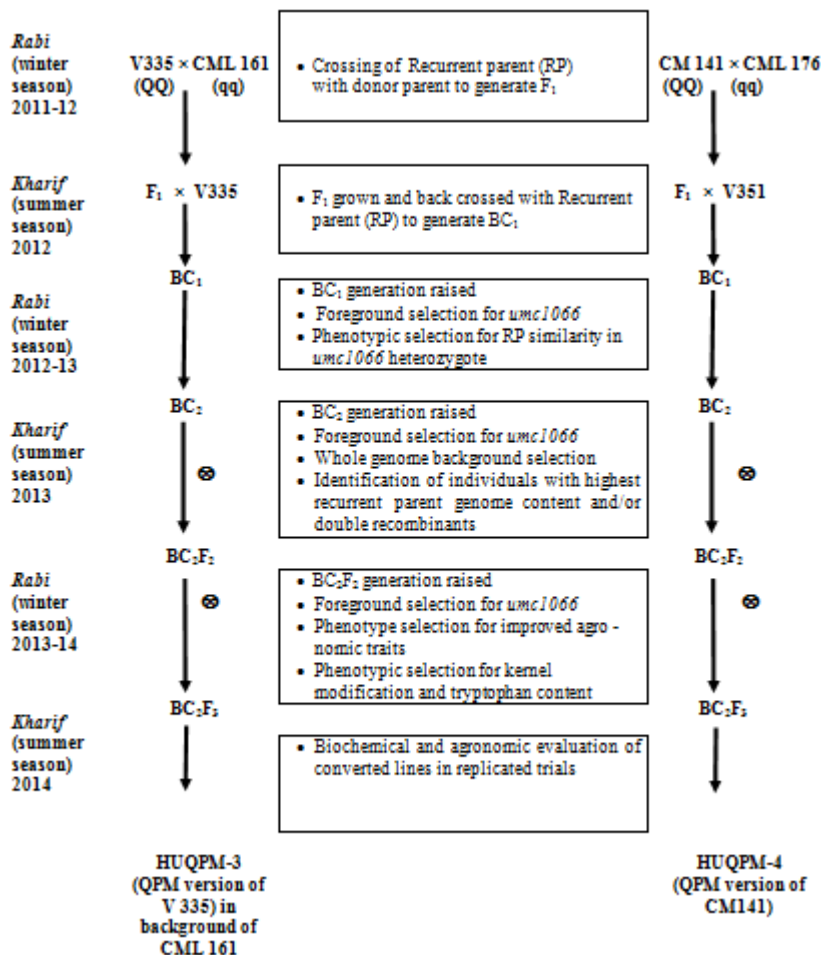


Figure 1: A schematic representation of simultaneous conversion of normal inbreds to Quality Protein Maize (QPM)

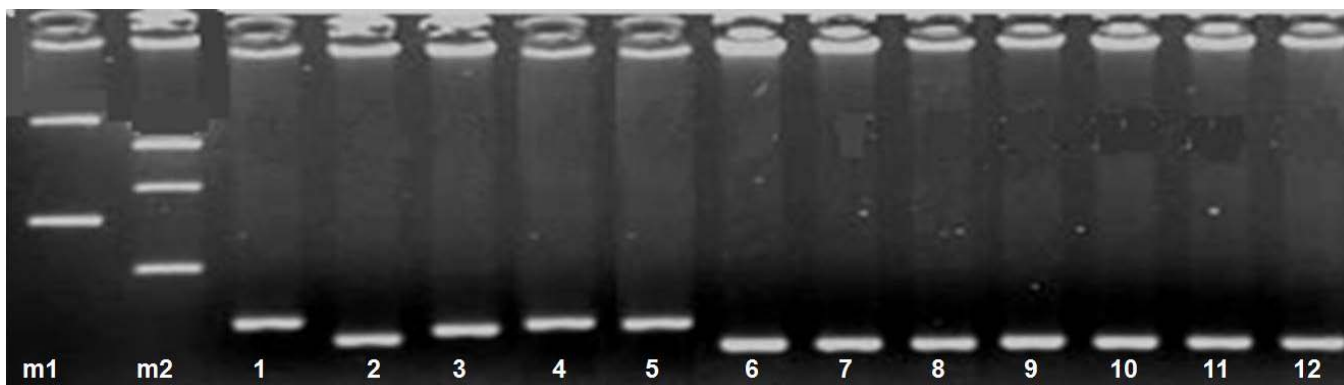


Figure 2: Parental Polymorphism Assay between Normal and QPM Inbreds. Parental polymorphism analysis using *Opaque 2* specific SSR marker *umc 1066* between normal and QPM inbred lines. Lane 1: m1 : 1kb marker, Lane 2: m2:100bp marker and Lane 3 to 14 as follow: 1- V 335, 2- CM 141, 3- CM 212, 4- V 361, 5- V 338, 6- V 351, 7- V 341, 8- V 336 (all normal inbreds) 9- CML 141, 10- DMR QPM 58, 11- CML 176 & 12- CML 161 (all QPM)

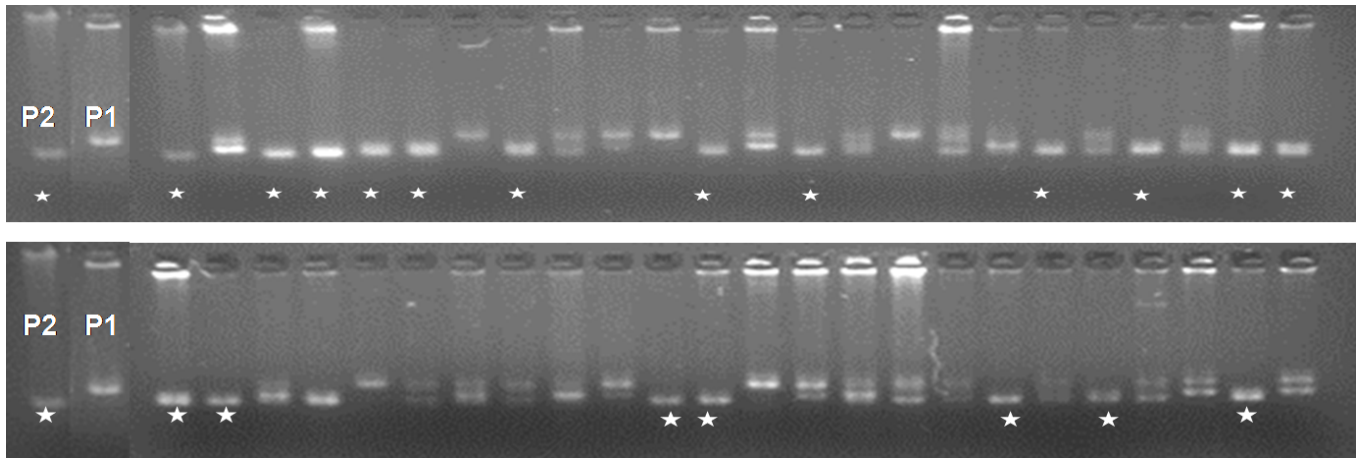


Figure 3: Identification of homozygous recessive individuals in BC₂ F₂ generation employing *umc 1066*. The first two lanes correspond to Donor Parent-QPM (P2) and Recurrent Parent-non QPM (P1) while rests of them are individuals of BC₂ F₂ population. The individuals indicated by * are homozygous for recessive *opaque-2* mutant allele