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Marker assisted introgression of *opaque* 2 allele for development of quality protein maize inbred lines in Sri Lanka

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Abstract

Maize (*Zea mays* L.) plays a very important role in human and animal nutrition in many developed and developing countries, worldwide. Tryptophan and Lysine content of maize, which is normally deficient, can be enhanced by transfer of the *opaque* 2 (O_2) gene along with several other modifier genes. Marker assisted breeding is the most effective and efficient way to develop quality protein maize (QPM). Crosses were made between recurrent parent CML 20 with different QPM donor parents CML 194, CML 193, CML 161, CML 164 and CML 171. The cross combination CML 20 / CML193 cross was selected for further study. According to the light table selection index, phenotypic selection for kernel modification was around 22-28% seeds out of BC_1F_2 population contributing to 2nd and 3rd categories were used to generate BC_2F_1 and BC_2F_2. Marker assisted selection was done using SSR marker *phi* 057 in BC_2F_2 generation. Out of 210 plants, 30 plants were identified with *opaque* 2 genes. Those thirty plants selected from BC_2F_2, were advanced to BC_2F_6 and then subjected to PCR amplification. The results, showed that 10 plants as homozygous for *opaque* 2 recessive allele. Selected 10 lines were further evaluated for yield and agronomic traits. Among the new QPM lines, MIQPM 6, and 9 showed a grain yield more than 4 t ha^{-1}, which was comparable with two CIMMYT QPM lines of CML 194 and CML 161. Except MIQPM 5, other tested lines showed acceptable grain yield about 3-4 t ha^{-1}. Shelling percentage of inbred lines varied from 70 -80%. Further, both newly developed MIQPM 6 and MIQPM 9 showed higher number of average tassel branches as eight compared to other lines. Thus, based on the yield, plant and ear characteristic's the new QPM lines, MIQPM 6 and MIQPM 9 can be identified as promising lines possessing O_2O_2 genotypes. Extensive

review of literature indicates that, this is the first report of a study in Sri Lanka where quality protein maize lines were developed using marker assisted breeding.

Keywords: Marker-assisted breeding, *Opaque* 2 gene, Quality protein, *Zea mays* L

Introduction

Maize (*Zea mays* L.) occupies the second largest extent of the total cultivated extent of cereals in Sri Lanka, where about 95% is rice, 4% is maize and the rest is occupied by millets (Kumari *et al.,* 2013). Maize plays a very important role in human and animal nutrition in Sri Lanka and a considerable amount of maize grains are utilized in the formation of weaning food for lactating mothers and for children. Maize endosperm consists of approximately 9 -12% of protein, but is naturally deficient in two essential amino acids; lysine and tryptophan, leading to poor biological value of normal maize varieties. A breakthrough came in the 1960s' with the discovery of the enhanced nutritional quality mutant *opaque* 2 (O_2) (Mertz *et al.,* 1964).

The *opaque* 2 (O_2) is a recessive gene located on the chromosome 7 and its modifiers behave as a multigenic trait (Jompuk *et al.,* 2006). Quality Protein Maize (QPM) is produced by incorporating modifier genes that convert the undesirable soft and chalky kernel to the hard endosperm. The O_2O_2 mutation and the modifiers/enhancers of lysine and tryptophan are not sufficient to develop an agronomically acceptable maize variety, which is high in lysine and tryptophan.

Marker -assisted selection (MAS) in combination with conventional breeding can greatly accelerate the introgression of modified *opaque* 2 gene in to elite maize lines. Microsatellite markers located within the O_2 gene have provided opportunities for accelerating the pace of QPM conversion programs through MAS.

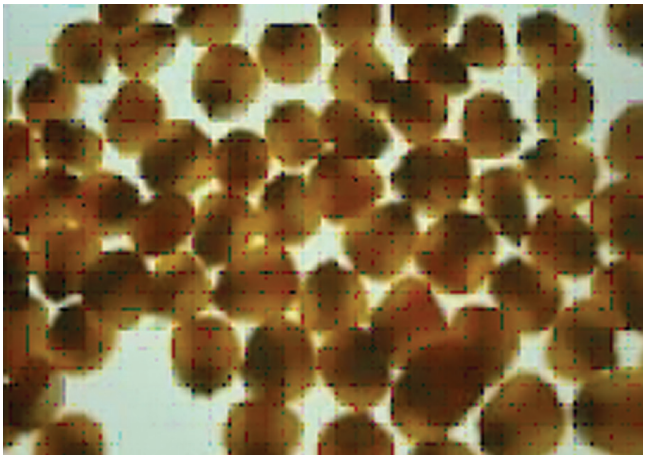
The present study was carried out with the objective of improving the protein quality of maize and hard endosperm using Marker Assisted Selection (MAS) and phenotypic selection.

Materials and methods

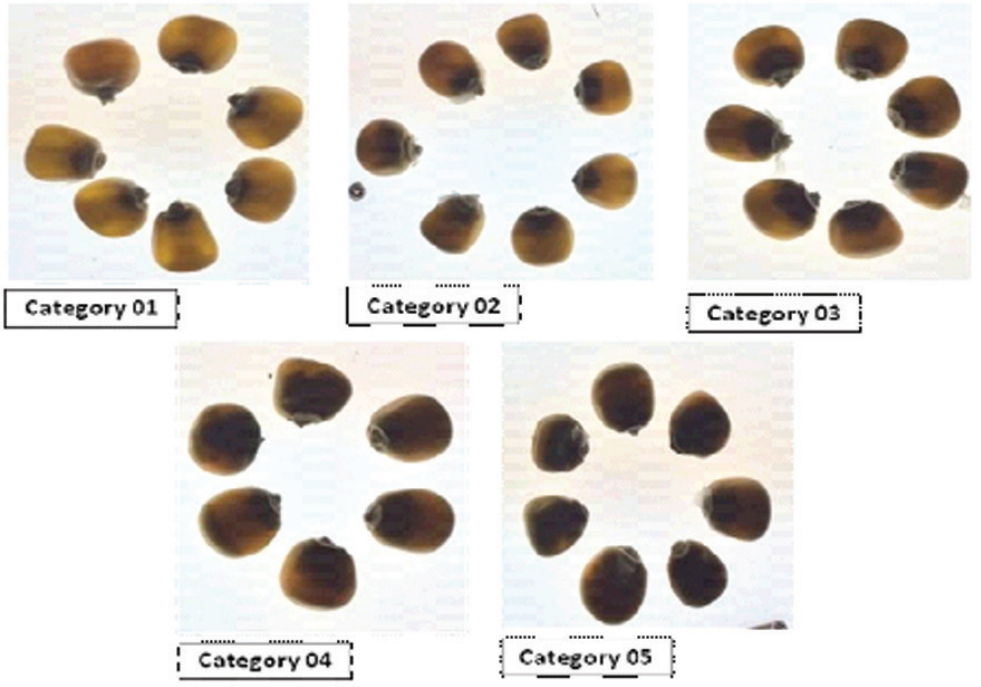
The study was carried out at field and the biotechnology laboratory of the Field Crops Research and Development Institute (FCRDI), *Mahailuppallama*, Sri Lanka during the seasons 2017 *Yala*, 2017/2018 *Maha*, 2018 *Yala*, 2018/2019 *Maha*. The soil of the experimental field consisted of Reddish Brown Earth. Agronomic practices including, fertilizer applications were done according to the recommendations of the Department of Agriculture (DOA). Surface irrigation was practiced when required. Line CML 20 was selected as the recurrent parent and CML 193, CML 161, CML 171, CML 164 and CML194 lines, which have *opaque* 2 (O_2) alleles, were used as the donor parents in back cross breeding program. The F_2 population was generated by selfing F_1 generation and then seeds were bulked and selected for next generation by using a locally made light table as described by Vivek *et al.* (2008) (Figure 1). The light table was made of wood on all sides except for the top surface, which was made of semi-transparent plastic. Light table helped to view kernel characteristics and differentiate hard endosperm maize types from soft O_2O_2 genotypes when seeds were placed on the surface of the table and the florescent light was turn on. Selected five lines (CML 20 / CML171, CML 20 / CML161, CML 20 /CML 193, CML 20/ CML 164, CML 20/CML194) were backcrossed with CML 20 where 50 crosses were made for each cross combination. Fifty plants from BC_1F_1 populations were advanced to BC_1F_2 population by selfing each other. Considering the field performance of BC_1F_2 population, CML 20 / CML 193 cross combination was backcrossed with recurrent parent CML 20 and 75 crosses were made for the QPM development program.

Evaluation of opaqueness of seeds

Maize kernel modifications were phenotypically evaluated using the light table selection index (Figure 1) described by Vivek *et al*. (2008). This technique was used to evaluate F_2, BC_1F_1, BC_1F_2, BC_2F_1, and BC_2F_2 populations. Seeds which were selected from light table were used to generate BC_1F_1, BC_1F_2, BC_2F_1 and BC_2F_2.



A



B

Figure 1. The level of seed opaqueness for kernel modification; Plate A. Seed lay down on the translucent sheet of light table showing degrees of opaqueness, Plate B. Seeds categorized according to the degree of opaqueness.

Molecular screening - Seeds, which were selected from light table, were planted and two to three weeks old leaf samples were collected from QPM parent (CML 193), non QPM parent (CML 20) and populations of BC_2F_2 generation. Leaf samples (100 mg) were prepared and stored in a minus 80°C freezer for further use. These samples were ground using chilled mortars and DNA extraction was done using a modified CTAB method (Allen *et al*., 2006). Isolated DNA was treated with RNase (Promega, USA) to remove RNA.

PCR amplification and detection of polymorphism - Molecular screening was done for the plants of selected BC_2F_2 (CML20 / CML 193) using co- dominant SSR maker *phi057.* Primer sequences of *phi057* (F= forward and R= reverse) used were as follows F5′-CTCATCAGTGCCGTCGTCCAT-3'and Reverse primer 5′-CAGTCGCAAGAAACCGTTGCC -3' (Danson *et al.,* 2006). The PCR amplification reaction was carried out with *phi057* in a 15 µl reaction volume for each DNA sample, containing 5X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, 25 U of *Taq* DNA polymerase (Promega, USA), 10 mM of each primer and 50 ng of template DNA using ABS Verity thermal cycler (Applied Bio Systems, USA). The 35 cycles of PCR consisted of denaturation at 94 °C for 5 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 4 min. The amplified product selection was done using polyacrylamide gel electrophoresis (PAGE (8% =acrylamide 29: 1 bis acrylamide). The gel was stained with 0.5µg/ml Ethidium Bromide and visualized on UV Trans illuminator (Quantum CX5, Germany).

Generation advancement and molecular screening of advanced lines of BC_2F_6 - Lines selected with *opaque 2* genes through PCR were advanced to BC_2F_6 by selfing at each generation and they were subjected to PCR confirmation to identify homozygous inbred lines for *opaque 2* recessive allele.

Field evaluation of promising inbred lines - During 2018 *Yal*a and 2018/2019 *Maha* seasons, 10 QPM inbred lines were evaluated using two parental lines CML20 and CML193, which were used for QPM inbred line development program with four other CIMMYT inbred lines of CML 194, CLYQ 203, CML 161, CLO 2450, local maize hybrid

01 and exotic maize hybrid Pacific 399. The evaluation of promising selected inbred lines was carried out in a randomized complete block design. The plot size used was 5 m length, and 1.2 m in width. DOA recommended fertilizer levels (Urea 325 kg ha^{-1}, Triple super phosphate 100 kg ha^{-1}, Muriate of potash 50 kg ha^{-1}) and recommended spacing 60 cm × 30 cm per hill were used. The Grain yield and other yield related agronomic traits, and plant characteristics were measured. Grain yield was measured on plot basis and adjusted to 15% grain moisture. Other plant characteristics were measured in six sub samples from each plot. Analyses of variance were performed with mean separation using Duncan's new multiple range test in Statistical Analysis Software (9.1 V).

Results and discussion

The present investigation was established to convert normal maize inbred lines to quality protein lines with high lysine and tryptophan content by using a marker assisted backcross breeding program. The *opaque 2* gene is recessive and modifiers are polygenic, therefore, incorporating of elite inbreds with donor inbred is not a straight forward procedure. In this study, two generation backcrosses were conducted for each cross to obtain homozygous recessive alleles. Phenotypic kernel modification was evaluated with the help of the light table selection index and agronomic traits in subsequent self-pollinated generations. Babu *et al*. (2005) and Gupta *et al.* (2009) reported similar conversions of normal maize to QPM types using three SSR molecular markers *Phi112, phi057* and *umc 1066*. However, in this study, parental line screening was done using *phi057* and polymorphism was clearly observed within QPM donor parent CML193 and recurrent parent CML 120 in molecular marker analysis.

BC_2F_1 was self-pollinated to obtain BC_2F_2 because kernels segregated for hardness at different levels of modifications. In the present study, molecular screening was done at BC_2F_2 generation. Phenotypic screening of individual kernels under transmitted light and selection of kernels that have less than 25% opaqueness is by far the most convenient strategy employed in the QPM breeding programs (Singh *et al.,* 2017). The *opaque 2* O_2) mutation and the modifiers/ enhancers of lysine and tryptophan are by themselves, not sufficient to develop agronomical acceptable maize high in lysine and tryptophan. Pleiotropic effects of the O_2 allele make the maize endosperm soft and susceptible to cracking, ear rots and weevils. Such negative secondary effects are obviously undesirable.

This softness expresses itself as an opaque phenotype that can be viewed on a light table. In this study a custom-made light table was used to pick out kernels with the *o2o2* genotypes by using the degree of opaqueness.

Category 1 consisted viz. 0% opaqueness seeds and such seeds were rejected, Category 2 consisted of less than 25% opaqueness, while category 3 consisted with 25% to 50% opaqueness and both category 2 and 3 were selected for generation advancement. Category 4 and 5 were rejected because no translucent nature in seeds and seed appeared in dull color (Figure 1). In F_2 generation level, kernel modifications were observed in each back cross and selfed families using light table index. Around 22- 28% seeds out of BC_1F_2, population contributed to 2nd and 3rd categories according to the light table selection index and they were advanced to generate F_2, BC_1F_1, BC_1F_2, BC_2F_1 and BC_2F_2 populations.

PCR amplification and detection of polymorphism

The marker *Phi057* was polymorphic for both parents. The polymorphism of two parents and 16 individuals of BC_2F_2 population and it's polymorphism were illustrated by figure 2. Therefore, molecular screening was done for phenotypically vigorous two hundred and ten individuals of BC_2F_2 population and 30 homozygous recessive plants for *opaque* 2 gene were selected for generation advancement.

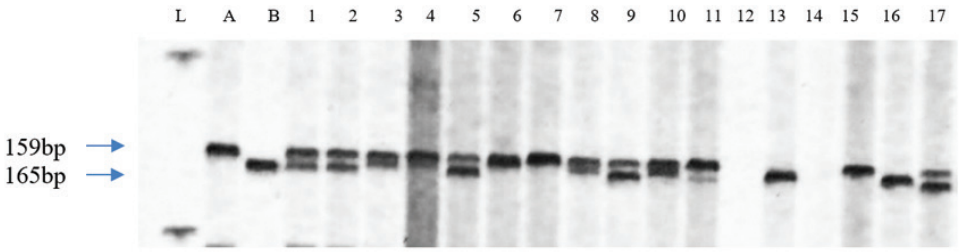


Figure 2. Identification of homozygous recessive individuals in BC_2F_2 generation by using SSR marker *Phi057*

L-100bp Ladder, A-CML20-recurrent parent, B-CML193- donor parent, 1-17 individuals of BC_2F_2 population. Line numbers 13and 16 were homozygous for opaque 2 recessive allele, line no's 1,2,3,4,5,8,9,10, 11 and 17 were heterozygous for opaque 2 recessive allele, line no's 6, 7 & 15 were dominant homozygous for recessive allele.

Molecular screening of advanced lines of BC_2F_6

BC_2F_6 advanced 30 lines were subjected to PCR amplification and 10 individuals were identified as homozygous for *opaque 2* recessive allele (Figure 3)

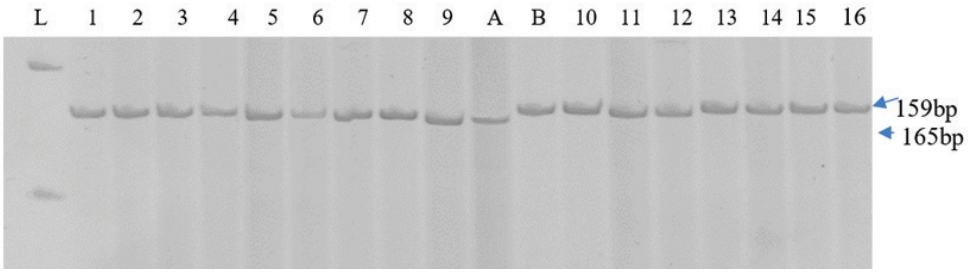


Figure 3. Selection of homozygous BC_2F_6 lines (CML 20 / CML 193) for *opaque* 2 genes

L – 100 bp ladder, A- CML 193- donor parent, B- CML20- recurrent parent, 1-9 homozygous for ***opaque*** *2 recessive allele and 10-16- dominant homozygous for recessive allele.*

Yield traits of QPM inbred lines

Table 1 showed the yield components of developed QPM lines, check inbred lines and hybrids. Analysis of variance showed significant differences ($p < 0.05$) among inbred lines for evaluated characteristics. Obviously F_1 check hybrids showed higher grain yield and other yield component traits compared to inbred lines due to hybrid vigor (Hallauer *et al*., 2010). MIQPM 6, and MIQPM 9, which were developed by this study showed the grain yield above 4 t ha^{-1}, and it was comparable with CIMMYT promising QPM lines of CML 194 and CML 161. Except MIQPM 5, other lines showed acceptable grain yield about 3-4 t ha^{-1}. Shelling percentage of inbred lines varied from 70 -80%. Further, QPM lines 3, 4, 6 and 9 showed higher 1000 grain weight (213 -235 g) compared to other new lines. Grain yield, thousand-kernel weight, ear length and ear circumference are important in determining the suitability of an inbred line as a seed parent in a seed production field (Pinnisch *et al*., 2012). The plant and ear traits of developed inbred lines showed higher variability for grain yield, thousand-kernel weight, ear length & ear circumference among them (Table 2). The check F_1 hybrids showed higher values for most of the plant and ear traits. The developed MIQPM lines 3, 8 and 9 showed taller plants with a height above 105 cm. Due to inbreeding depression maize inbred lines showed lower plant height and less vigorous growth compared to hybrids or open pollinated populations (Shull, 1908). However, inbred lines that are relatively taller with strong stem are advantageous when selecting as parents for hybrid development (Shull,

1908). Inbred lines with longer tassel and higher tassel branches can be used as male parents (Pinnisch *et al*., 2012). The newly developed QPM lines 6 and 9 showed higher number of average tassel branches (8) compared to other new lines. However, promising CIMMYT inbred line, CML 161 showed the 14 tassel branches. Thus, based on the yield, plant and ear characteristic's the new QPM lines, MIQPM 6 and 9 can be identified as promising lines possessing *0202* genotypes.

Table 1. Average grain yield and yield components of Maize QPM inbred lines evaluated in 2018 *Yala* and 2018/19 *Maha* at FCRDI, *Mahailluppallama*.

Line	Average grain yield (t ha^{-1})	Shelling (%)	Grain weight of 5 ears (g)	Grain weight per ear (g)	1000 grain weight (g)
MIQPM 1	3.1 ^{cde}	0.8^{b}	271^{cde}	54.2^{cde}	192^{cdef}
MIQPM 2	3 ^{fcde}	0.8^{bc}	265^{fcde}	53^{fcde}	146^{gf}
MIQPM 3	3.5^{bcde}	0.8^{bc}	314^{bcde}	62.8^{bcde}	213^{cde}
MIQPM 4	3.1^{cde}	0.7^{e}	272^{cde}	54.4^{cde}	216^{cde}
MIQPM 5	2.6^{fde}	0.8^{bcd}	228^{fde}	45.6^{fde}	186^{cdef}
MIQPM 6	4.4^{bcd}	0.8^{bced}	389^{bcd}	77.8^{bcd}	235^{cb}
MIQPM 7	2.8^{fcde}	0.8^{ced}	246^{fcde}	49.2^{fcde}	172^{ef}
MIQPM 8	3.7^{bcde}	0.8^{bced}	329^{bcde}	65.8	185^{cdef}
MIQPM 9	4.1^{bcde}	0.8^{bced}	361^{bcde}	72.2^{bcde}	223^{cdeb}
MIQPM 10	3.2^{cde}	0.8^{b}	279^{cde}	55.8^{cde}	169^{ef}
CML 193 *	4.1^{bcde}	0.7^{ed}	363^{bcde}	72.6^{bcde}	211^{cde}
CML194*	5.1^{b}	0.8^{bced}	452^{b}	90.4^{b}	220^{cde}
CML20**	3.5^{bcde}	0.7^{e}	312^{bcde}	62.4^{bcde}	192^{cdef}
CLYQ203*	2.4^{fe}	0.7^{e}	210^{fe}	42^{fe}	178^{def}
CML161*	4.5^{bc}	0.8^{b}	394^{bc}	78.8^{bc}	229^{cdb}
CLO2450**	1.3^{f}	0.7^{f}	115^{f}	23^{f}	112^{g}
CML451**	3.1^{cde}	0.8^{b}	272^{cde}	54.4^{cde}	228^{cdb}
MI Maize HY 1 (local hybrid)	8.6^{a}	0.8^{bc}	763^{a}	152.6^{a}	274^{ab}
Pacific 399 (imported hybrid)	9^{a}	0.9^{a}	819^{a}	163.8^{a}	298^{a}
CV %	32	4	32	18	18
Mean across inbred lines	3.4	0.8	59.7	194.5	194.5
Mean across hybrid lines	8.8	0.85	158.2	286	286.0

*CIMMYT QPM lines, ** CIMMYT normal lines, Mean values with same letters are not significantly different at $p<0.05$

Table 2. Seasonal average and standard deviation of plant and ear characters of maize QPM inbred lines.

Entries	PH (cm)	TL (cm)	TB No.	NL No.	ELL (cm)	ELW (cm)	E/P number	EL (cm)	EC (cm)
MIQPM 1	84±3.4	30±0.8	5±0.4	10±0.2	55±1.6	7±0.2	1±0.0	13±0.4	11±0.2
MIQPM 2	93±3.5	28±0.7	5±0.4	10±0.2	50±1.6	6±0.2	1±0.1	12±0.3	11±0.2
MIQPM 3	104±3.3	33±1.1	6±0.4	10±0.2	57±1.9	6±0.1	1±0.0	13±0.6	12±0.2
MIQPM 4	89±2.9	34±1.3	7±0.4	10±0.2	54±1.8	6±0.2	1±0.1	13±0.4	12±0.1
MIQPM 5	79±2.6	32±1.0	5±0.4	9±0.2	50±1.1	6±0.2	1±0.0	12±0.5	11±0.1
MIQPM 6	98±1.9	30±1.5	8±0.4	10±0.4	54±1.5	7±0.2	1±0.0	13±0.7	13±0.2
MIQPM 7	96±2.8	28±0.8	5±0.5	10±0.2	52±2.4	6±0.2	1±0.0	12±0.6	12±0.2
MIQPM 8	107±3.5	29±0.9	7±0.4	10±0.2	57±2.2	6±0.3	1±0.1	12±0.4	12±0.2
MIQPM 9	112±2.9	31±0.9	8±0.5	11±0.2	59±1.8	7±0.2	1±0.1	13±0.5	12±0.3
MIQPM 10	90±1.9	32±0.7	4±0.3	10±0.3	51±1.5	6±0.2	1±0.1	13±0.4	11±0.2
CML 193 *	106±3.4	34±0.8	8±0.5	11±0.2	57±2.0	7±0.3	1±0.1	14±0.5	13±0.6
CML194*	93±2.1	34±0.6	9±0.4	11±0.3	60±1.5	7±0.2	1±0.1	15±0.4	13±0.1
CML20**	113±3.6	35±0.9	7±0.5	11±0.3	62±2.0	7±0.2	1±0.1	17±3.6	13±0.2
CLYQ203*	81±2.5	26±0.8	5±0.3	11±0.2	48±1.4	7±0.2	1±0.0	11±0.4	12±0.2
CML161*	106±3.5	30±1.0	14±0.9	11±0.2	59±2.5	8±0.2	1±0.1	13±0.5	13±0.2
CLO2450**	81±4.1	31±0.8	7±0.7	10±0.4	59±1.9	6±0.2	1±0.0	11±0.5	11±0.2
CML451**	104±2.4	29±1.0	10±0.5	11±0.4	53±2.0	8±0.2	1±0.0	14±0.5	12±0.3
MI Maize HY 1 (local hybrid)	135±4.0	38±0.7	19±0.7	15±2.5	65±2.5	8±0.2	1±0.1	19±0.4	14±0.2
Pacific 399 (exotic hybrid)	121±2.6	39±0.9	8±0.4	11±0.2	66±4.0	8±0.2	1±0.1	16±0.3	15±0.1
CV%	12.5	13.6	35	36	15	15	32	38	9.8
Mean across inbred lines	96.2	30.9	7.1	10.4	55.1	6.6	1.0	13.0	12.0
Mean across hybrids	128.0	38.5	13.5	13.0	65.5	8.0	1.0	17.5	14.5

**CIMMYT QPM lines, ** CIMMYT normal line, PH – Plant height (cm), TL – Tassel length (cm), TB – No. of tassel branches, NL – No. of leaves at flowering, ELL – Ear leaf length (cm), ELW – Ear leaf width, E/P – No. ears per plant, EL – ear length, EC- Ear circumference (cm).*

Conclusion

Genotyping of BC_2F_6 generation revealed that 10 lines were homozygous for *opaque* -2 recessive alleles and further light table data also proved it by showing required degree of opaqueness. To observe the expression of *opaque* 2 recessive alleles, measurement of tryptophan and lysine is recommended. New QPM lines, MIQPM 6 and MIQPM 9 gave the comparable yield 4 t ha^{-1}.Thus, based on the yield, plant and ear characteristic's the new QPM inbred lines, MIQPM 6 and MIQPM 9 can be identified as promising lines for selecting as parents. Extensive review of literature indicates that, this is the first report of a study in Sri Lanka where quality protein maize lines were developed using marker assisted breeding.

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