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Full Length Research Article

Molecular and Morphological Characterization of *Zea mays* L. Genotypes for Opaque 2 Gene and Yield Contributing Traits

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Abstract

Maize (*Zea mays*) is an important cereal crop grown insubtropical and temperate climates where annual rain fall250 mm to 5000 mm per year. In the present study, 50 maize inbred lines were characterized for molecular and yield contributing traits. Fifty inbred lines were screened for the presence of *opaque 2* gene. Molecular characterization showed thatL24, L25, L31 and 40 genotypes were heterozygous for the locus, confirmed byby amplifying 169 bp in QPM hybrid and 161 bp in inbred lines. Analysis of variance and principal component analysis (PCA)showed the presence of wider diversity in maize inbred lines. There were highly significant differences present in inbred lines for all the parameters studied,the correlation among different traits were also determined, and it was found that plant height and ear height had highly significant positive correlated with yield per plant, ear diameter and the number of grains per row had a significant positive correlation with yield per plant.

Key words: *Zea mays*, bio-fortification, *Opaque-2* gene, PCA analysis.

1 Introduction:

According to FAO (2015), 794.6 million people in the world are suffering from malnourishment which is prevailing at a rate of 10.9 percent. Out of 794.6 million people in the world, 779.9 million malnourished people belong to developing countries with 511.7 million only in Asia, including Pakistan, and 14.7 million people are from developed countries. Food security is the biggest issue of the present time that required extensive work to achieve the objectives of food security that food should be available, accessible and of good quality and quantity (Raza *et al.,* 2020).

Cereal crops cover 94% of the world production cultivated for both food and feed purpose with major crops wheat, rice and maize (Ranum*et al*., 2014). Cereals can provide approximately 50% protein and have the potential of 70%. Still, this protein is not the balanced protein as all cereal crops are deficient in essential amino acids such as lysine and tryptophan that ranges from 1.5 to 2% and 0.25 to 0.5% respectively whereas the requirement for the optimal human diet is 5% lysine and 1.1% tryptophan (Gibbon *et al*., 2005 and young *et al*., 1998). Deficiency of lysine and tryptophan is responsible forkwashicore disease in children which is characterized by growth failure, fatty liver, adema and irritability (Vivek, 2008).

Maize (*Zea mays* L.) was one of the firstcultivatedplants having a centre of origin Mexico grown subtropical and temperate region and having C4 physiology (Ranum*et al*., 2014)). In Pakistan maize is the third most important cereal crop and is consumed as a staple food in many countries of the world like USA and African countries and also cultivated for livestock feed that fulfils the requirements of protein, minerals and starch. In Pakistan maize is two seasonal crops cultivated in autumn and spring that cover the cultivation of 80% and 20% respectively. In Pakistan,35% of total production is used as feed, 23% as food and 31% for other uses (Paroda*et al*., 2015). It shares 2.2 % value-added in agriculture 0.4% to GDP. The area under maize cultivation is 1144 thousand hectares, and production is about 4.920 million tonnes (Anonymous, 2015-16).

In USA many maize mutants*opaque-2* (*o2*), *floury-2* (*fl2*), *opaque-7* (*o7*), *opaque-6* (*o6)* and *flouri-2* (*fl2*) were identified causingthe high level of lysine and tryptophan in maize grain, among these mutations,*opaque 2* can be easily manipulated during

the breeding program, and its presence in homozygous form doubles the amount of lysine and tryptophan (Crow and Kermicle, 2002; Vivek, 2008). *Opaque 2* is a regulatory mutation that directly effects the transcription of zein mRNA by down regulating it (Soave *et al*., 1978; Kodrzyck*et al*., 1989; Munk, 1992).It has been observed that proportion of zein protein is less and non-zein protein is more in *opaque 2* mutant lines maintaining the total protein of the grain at the same level, and therefore this mutation is not directly involved in the synthesis of both amino acids because tryptophan showsa significant negative correlation with zein protein (Vivek, 2008; Olakojo*et al*. 2007).

Opaque 2 mutation also produces pleiotropic effects like soft and chalky endosperm that causes undesirable phenotype leading to low yield, susceptible to storage pest and ear rot. The interdisciplinary research team at International Maize and Wheat Improvement Centre (CIMMYT), Mexico developed hard endosperm from floury soft endosperm through conventional breeding techniques having same yield potential, resistant to the insect pest, storage and utilization quality superior to non-opaque 2 maize lines. This agronomically acceptable and nutritionally improved opaque-2 maize developed at CIMMYT was termed as "Quality Protein Maize" or QPM (Vivek *et al*., 2008). QPM has potential to meet 100% requirement of lysine and tryptophan by consuming 100g/day for younger and $500gday^{-1}$ for adults. In a meta-analysis of nine community base studies showed that there is a 9% increase in growth rate in height and 12% increase in the growth rate of weight (Gunaratna *et al*., 2010).

Simple sequence repeats (SSR) or microsatellite are a genomic region which consists of highly variable tandem repeats with two to six DNA nucleotides (Jacob *et al*., 1998; Raza *et al.*, 2019). These SSRs are used as flanking primers for the amplification of the desired gene using PCR (Saiki *et al*., 1988). The amplified products are separated electrophoreticallyfollow mendelian inheritance, behave as a co-dominant genetic marker and are highly polymorphic (Backman and Soller, 1990).

Yield contributing traits like 100-grain weight, yield per plant, cob diameter, cob length, etc. play an important role in selecting maize genotypes for variety development and are essential for the breeding program and should be taken into account either simultaneously or alone for selecting high yielding genotypes (Ranawat*et al*., 2013). For developing a commercial QPM hybrid, there must be a procedure study on the characterization of genotypes for agronomic traits (Sood*et al*., 2017). For evaluation of a large number of genotypes in the breeding program, statistical tools like D^2 and principalcomponent (PCA) analysis are used for checking the genetic potential and genetic divergence among genotypes.

Keeping in view the above-mentioned facts, the current study was conducted with the following objectives; to access variability among maize inbred lines for various agro-morphological traits and the presence of *opaque 2*to access variability and performance of inbred lines in Pakistani climate.

2 Material and methods:

The proposed study was carried out to evaluate the presence of *opaque 2* gene and genetic diversity between 50 maize genotypes. The said research consists of two analyses. The first analysis conducted for screening the 50 maize genotypes for *opaque 2* gene at seedling stage using three *opaque 2* specificSSR primers. Plant material consists of 50 maize genotypes that were taken from Pakistan Genetic Resource Institute (PGRI) and 12 QPM hybrids from CIMMYT. Genotypes were grown in the field of University of Agriculture, Faisalabad in Randomized Complete Block Design with two replications in 5th of August 2016.

2.1 Molecular analysis

Leaf sample was taken from the four weeks-old seedlings, and genomic DNA was isolated according to the standard protocol of GMO testing lab of Agriculture Biotechnology Research Institute, AyubAgriculture Research Institute, Faisalabad. The protocol was as follows: leaf tissue was ground to a fine powder in liquid nitrogen using pre-chilled autoclaved pastel-mortar. Approximately 1 g of ground material was digested with 2XCTAB for the breakdown of the cell membrane and cell wall and releasing of nucleic acid. Phase separation of nucleic acid was done using chloroform, and isoamylalcohol (24:1) and precipitation of nucleic acid was carried out using chilled iso-propanol. RNA was digested using 5µL*RNAase* and incubated at 37ºC for 1 hour, followed by phase separation and DNA precipitation with 100% ethanol. DNA pellet was dried and dissolved in 200 µl d_3H_2O. DNA quantification was done by using the spectrophotometer (Nano drop 2000) and working dilutions ($15\ ng\mu L^{-1}$) was made to perform PCR.

SSR analysis was done using to *o2* specific primers; the (*phi 057*) co-dominate primer and, the (*phi 112*) dominate primer. Primers were bought from Gene

link. Primer sequences, their annealing temperature and amplified products are given in Table 1.

PCR products of *phi112* and *phi057* were separated on 2% agarose and 6% acrylamide gels respectively with 0.5X TBE buffer. Agarose gels were pre-stained with ethidium bromide (0.5 µgµL^{-1}), and Acrylamide was post stained with $AgNO_3$(0.0018µgµL^{-1}). Gel bands were visualized and documented on transilluminator and gel documentation system. SSR profile was scored as presence (*phi 057*) or absence (*phi 112*) of fragments in each sample.

2.2 Morphological analysis

For morphological analysis, three plants from each replication were studied. Parameters that recorded at maturity for inbred lines were as follows: days to 50% silking, days to 50% tasselling, plant height (cm), ear height (cm), no. of ears per plants, number of grains per ear, number of grain per row, ear diameter (mm), ear length (cm), 100 seed weight (g), yield per plant (g), anthesis to silking interval (ASI). Analysis of variance was performed for mean values of accessions for each replication and character. Significance of difference among all characters tested as described by Steel *et al*. (1997).Correlation analysis and PCA analysis were estimated using software XL-STAT 2016 to group the genotypes into homogenous classes. (Rencher, 1995; Weinstein, 2008).

3 Results and discussion

The increasing rate of malnourishment due to shortage of quality foods leads to many efforts that are being made to overcome it through bio-fortification. Deficiency of quality protein which is characterized by the deficiency of essential amino acids is also a major type of malnourishment. From the 1960s to onwards, there are many milestone achievements in maize bio-fortification for quality protein after the discovery and development of high lysine and tryptophan mutant in maize, the *opaque 2*mutant. Efforts are also being made in Pakistan for the evaluation of genotypes for opaque 2 gene and various agro-morpho traits that contribute towards higher-yielding. For the development of new verities, germplasm collection and evaluation are essential components of a breeding program.

3.1 SSR analysis

The present research demonstrates screening of 50 inbred lines using SSR for *opaque 2* gene. Although three gene-specific SSR primers were used, only *phi 057* has produced polymorphic results between *opaque 2* and non-*opaque* lines. The result from this primer was sufficient for the screening of *opaque 2* gene as the primer *phi 057*being co-dominant can differentiate between homozygous and heterozygous genotypes as two bands of equal intensity were considered as heterozygous for the locus and with one band as a homozygous for the locus. Our results show that primer *phi112*was amplified in all 50 maize inbred lines and a null allele in nine QPM hybrids out of 12 (Fig. 1 & 2). Primer *phi 057* amplified band sizes of approximately 161 and 169bp in all maize inbred lines (Fig. 3 and Fig. 4) and revealed differences between *opaque2* and *non-opaque 2* lines by amplifying the band size of around 161bp in non-opaque lines and a band size of approximately 169 bp in QPM lines.Lines 24, 25, 31 and 40 have opaque 2 allele in heterozygous form. Studies of Danson *et al* (2006) showed that *phi057* amplified a band size of 165bp for *opaque 2* lines and of 159bp for non-*opaque 2*lines. Whereas the studies of Babu *et al*. (2005) and Bantte and Prasanna(2003)showed that opaque 2 lines gave a band size of about 170bpand in normal lines amplification of a band size of approximately 160bp. This marker can be recommended for distinguishing between homozygous and heterozygous backcross progeny during the development of QPM hybrids. In the present study,amplified fragments in 9 QPM hybrids and 50 inbred lines with band size ranged from 160 to 170 bp respectively. These QPM specific primers could be used for foreground selection usingmarker-assisted selection (MAS) for the identification of desired genes without extensive phenotypic selection.

3.2 Analysis of variance

All the statistical analysis performed on various agro-morphological traits had revealed the presence of significant variation for all the traits of inbred lines **(Table II**). The traits that differ highly significantly were plant length, ear length, 50% tasselling, 50% silking, anthesis to the silking interval, ear length, ear diameter, number of ears, number of rows per ear, number of grains per row, yield per plant and 100-grain weight. Various lineswerefound to have the highest yield contributing traits that could be exploited for the development of higher yield maize hybrids. Ghimire (2015) also determined highly significant differences for these traits. High variability for agronomic traits of the germplasm used in the present study was supported by findings of previous studies (Dijak*et al*., 1999; Ihsan *et al*., 2005; Shrestha, 2013).

3.3Principal component analysis

The computed eigenvalues of twelve variables of 50 inbred lines were subjected to principal component analysis; their variability % and cumulative explained variance are present in the following tables. Eigen values of twelve principalcomponents had been shown in screw plot (Fig. 5). Following the proportion of variance criterion data revealed that out of eleven, five PCA describing more than one eigen values contributed 77.26% of the variation and were selected for further explanation (Table III). The principal component analysis resulted in the reduction of 12 original variables to five independent principal component variables. Variablesplant height, ear height, 50% silking, 50% tasselling, ear diameter, ear length, yield per plant, 100-grain weight, number of rows per ear and number of grains per row were noted as characteristics of variability (Table III).

First principal component showed most of the variability was caused by plant height, ear height and yield per plant. These traits had an important role in the variation of PC1 cluster that accounts for 64.15%. In PC1 maize genotypes were poor in ear length, 50% tasselling, 50% silking, number of rows per ear and number of grains per row. In the second principal component, 50% silking, anthesis to the silking interval, the number of grains per row and number of grains per ear exhibited more variability and contributed78.20% variation. In the third principal component traits that are contributing 75.02% variation are 50% tasselling and silking, ear length and ear diameter. In contrast,genotypes of maize are poor in plant height, ear height and ear length. The fourth principal component can be considered as a component of the number of ears, the number of rows per ear, 505 tasselling and anthesis to the silking interval that had an important role in producing 76.65% variation. Traits like plant height, ear height, 100-grain weight and 50% silking were poor attributes in the genotypes of PC4. According to fifth principal component 100 grain weight, the number of ears anthesis to silking interval accounts for 82.70% variation in the total variability of PC5 genotypes. Maize genotypes in the PC5 were poor in plant height, ear height, ear diameter, ear length and 50% silking (Table IV).

Contribution of PC1 towards variability was highest (24.33%) followed by PC2, PC3, PC4 and PC5 which contributed 17.64%, 13.86%, 10.42% and 10.09% variability respectively (Table IV). In PC1 and PC2 together 50% silking, anthesis to the silking interval, 50% tasselling, plant height, ear height, ear diameter, ear length, yield per plant, 100-grain weight and number of grains per row's differences are well represented in the biplot, but the number of ears and numbers or rows per ear have minimum differences between PC1 and PC2 biplot (Fig. 6). In summary, the results of PCA analysis showed that all traits contribute differently in total variation. These variations are sources of germplasm improvement in the respective quantitative trait which contribute toward better agronomic performance and increase in yield potential.

A principal component scatters plot of maize germplasm depicts those genotypes which are similar in their performance with respect to twelve variables. At the same time,genotypes which are further apart from one another are different. Genotypes which are nearer to variable vectors are more important for that trait and vice versa. Therefore scatter plot helped in the selection of genotypes for the yield contributing trait or for the trait that helped in better agronomic performance. In a given biplot it as clearly indicated that genotypes 14943, 14894, 14906, 14941 had higher 100-grain weight among 50 maize lines. Genotypes 14895 and 14938 had more yield per plant. Genotypes 14855, 14882, 14901 and 14952 had more number of rows per ear. For the selection of flowering traits, 14927, 14945, 14922, 14924, and 14923 genotypes are more important (Fig. 6).

Visual comparison among maize genotype could be accessed through genotype by trait (GT) biplot using multiple traits that analyze association among maize traits. Based on multiple traits GT biplot could be used for the independent culling thereby making selection effective by culling undesired genotypes enhancing better parent and cultivar selection (Yan and Rajcan, 2002; Yan and Tinker, 2005).

Biplot in the principal component exhibits variables that are super imposed on a plot as vectors where the relative length of vectors represents the relative presence of variability in each variable represented on the biplot. It also shows the association between two traits, if the angle between vectors of two traits is < 90° both are positively correlated whereas if the angle is>90° there is negative correlation and both vectors show no correlation if the angle is 90°(Sabaghnia*et al*., 2011).In the biplot graph, it was clearly indicated that theirplant height and ear height had strong positive correlation among them.Therefore plant height and cob height are

positively correlated like the results of Golam*et al*. (2011). Although most authors (Alvi*et al*. (2003); Najeeb *et al*. (2009); Bocanski*et al*. (2009) found strong correlative relation between plant height and ear height. Plant height also found to have a significant correlation with yield per plant, the same results were also determined by Golam *et al*. (2011), but it was highly significant. Singha and Prodhan (2000) also reported that grain yield is positively associated with plant height. Plant height also positively and significantly associated with 50% silking;these results were in agreement with Iqbal *et al*. (2015). Positive significant was also observed in ear diameter, and plant height, ear diameter and yield per plant same results were also observed by Iqbal *et al*. (2015), but these results were highly significant. Numbers of grains per row had highly and positive correlation with anthesis to the silking interval, these results were in contradicts with Iqbal *et al*. (2015). Days to 50% tasselling had a significant positive correlation with days to 50% silking likes the results of Golam*et al*. (2011) their results were highly significant. Simple correlation analysis showed that there was a highly significant positive correlation of days to 50% silking and anthesis to silking interval same results were also found by Iqbal *et al*. (2015).

Based on this study of inbred lines of maize PC1 was best for plant height (PH) and ear height (EH) and yield per plant PC2 was best for days to 50% tasselling, days to 50% silking, anthesis to silking interval and for the number of grains per row. PC3 was best for days to 50% tasselling and number of grains per row whereas PC4 and PC5 had characteristics features of numbers of ears, number of rows per ears and number of ears and 100-grain weight respectively. Overall in the 50 genotypes plant height (PH) and ear height (EH) and flowering traits are favorable characteristics for inbred lines (Fig. 6). Also, yield per plant (YPP), number of grains per row (NOGPR) and 100-grain weight (100Gwt) are major yield contributing that should be considered for the variety development in a breeding program.

Conclusion

Findings from this study show that more research works are required to understand the basics of QPM breeding program.As there was no opaque 2 gene in homozygous form among 50 genotypes, therefore,breeding of maize genotypes with high level of tryptophan requiredselfing of L24, L25, L31 and L40 for manipulation ofopaque 2 gene in local genotypes through appropriate conventional and non-conventional breeding techniques or by introducing QPM lines from CIMMYT for quality protein bio-fortification to alleviate protein malnutrition in the country. We found here following lines 14876, 14913, 14943 and 14923 for maximum 100-grain weight, yield per plant, number of rows per ear and number of grains per row respectively and hence can be used in the breeding of high yielding maize hybrids.Presence of a high level of genetic diversity in yield contributing traits during the current study could be used for maize germplasm characterization, conservation and improvement.Correlation plays a pivotal role in the selection of the desired combination of traits for breeding purposes. Different quantitative traits preferably plant height, ear height, 100-grain weight, number of grains per row and number of ears per row or the combination two or more can be useful for breeding programs.

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Figures Legends:

Fig. 1: SSR profilingof QPM hybrids

Fig. 2: SSR profilingof inbred lines

Fig. 3: SSR profiling of inbred lines

Fig. 4 SSR profilingof inbred lines

Fig. 5: Eigen value and cumulative variability of principle components

Fig. 6: Two-dimensional orientations of inbred lines for different traits on principle component biplot axis 1 and 2. Where 50%S = days to 50 % silking, 50%T = days to 50% tasselling, ASI = anthesis to silking interval, NORPE = number of row per ear, NOE = number of ears per plant, 100GW = 100 grain weight, PH = plant height, CH = cob height, ED = ear diameter, YPP = yield per plant and NOGPR = number of grain per row.

Table I: The essential information of SSR markers used in this st

Primers	**Sequence**	**Annealing T (ºC)**	**PCR Product(bp)**
Phi057	F: 5'-CTCATCAGTGCCGTCGTCCAT-3'	61.2	160-170
	R: 5'-CAGTCGCAAGAAACCGTTGCC-3'	61.6	
Umc1066:	F: 5'-ATGGAGCACGTCATCTCAATGG-3'	58.7	140-160
	R: 5'-AGCAGCAGCAACGTCTATGACACT-3'	62	
Phi112:	F: 5'-TGCCCTGCAGGTTCACATTGAGT3'	62.9	151
	R: 5'-AGGAGTACGCTTGGATGCTCTTC- 3'	60.7	

Table II: ANOVA for agro-morphological traits of inbred lines

S.O.V	**D.F**	**PH**	**EH**	**50%T**	**50%S**	**ASI**	**EL**	**ED**	**NOE**	**NORPE**	**NOGPR**	**NOGPE**	**100 Gwt**	**YPP**
Blocks	1	0.0002	71.85	5.76	6.76	25	1.77	45.79	0.04	0.87	34.81	12658.8	20.50	127.39
Lines	49**	11.87**	609.0**	109.0**	76.55**	35.2**	11.87**	59.53**	0.1**	7.84**	111.59**	22105**	70.75**	1062.2**
Error	49	2.165	4.75	6.90	5.43	6.5	2.165	4.53	0.0195	0.427	1.13	562.7	1.07	6.58

(NS= if p value>0.05, ** = p value < 0.01, * = p value > 0.01 or < 0.05), Where PH = plant height, EH = ear height, ED = ear diameter, EL = ear length, YPP = yield per plant, 100 Gwt = 100 grain weight, NOE = number of ears, NORPE = number of rows per ear, NOGPR = number of grains per row, 50%T = tasseling, 50%S = 50% silking, ASI = anthesis to silking interval.

Table III: Cumulative variances and EigenValues for principal component (1-5)

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Eigenvalue	3.030	2.412	1.931	1.444	1.228
Variability (%)	23.304	18.551	14.851	11.109	9.448
Cumulative %	23.304	41.855	56.706	67.815	77.263

Where PC1 = principle component 1, PC2 = principle component 2, PC3 = principle component 3, PC4 = principle component 4, PC5 = principle component 5.

Table IV: Contribution of variables in total variability in (PC1-PC5) of inbred lines

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PH	28.473	0.006	0.153	0.098	0.361
EH	23.783	0.103	0.436	0.776	0.605
ED	11.025	3.729	5.299	4.869	0.866
EL	3.391	6.768	2.544	29.072	0.851
YPP	13.978	6.110	3.767	8.880	3.828
100Gwt	6.557	0.150	5.642	0.157	25.372
NOE	1.138	0.755	5.055	31.450	25.421
NORPE	0.006	2.664	7.860	17.040	18.633
NOGPR	1.737	10.324	31.218	4.162	3.445
50% T	2.968	15.370	23.549	0.962	6.874
50%S	5.001	33.987	5.650	1.753	0.008
ASI	1.943	20.035	8.828	0.781	13.737

Where PH = plant height, EH = ear height, ED = ear diameter, EL = ear length, YPP = yield per plant, 100 Gwt = 100 grain weight, NOE = number of ears, NORPE = number of rows per ear, NOGPR = number of grains per row, 50%T = tasseling, 50%S = 50% silking, ASI = anthesis to silking interval, PC1 = principle component 1, PC2 = principle component 2, PC3 = principle component 3, PC4 = principle component 4, PC5 = principle component 5.

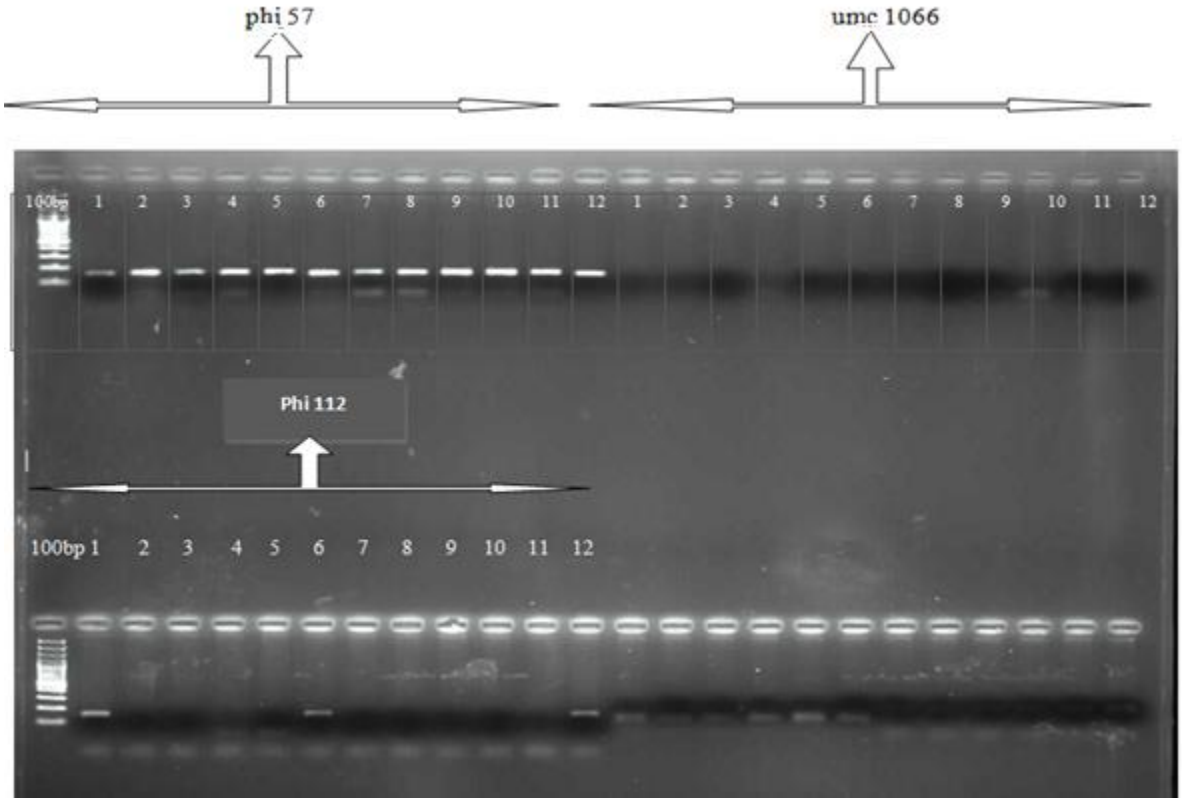

phi 57
umc 1066
100bp 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12
Phi 112
100bp 1 2 3 4 5 6 7 8 9 10 11 12

Fig. 1

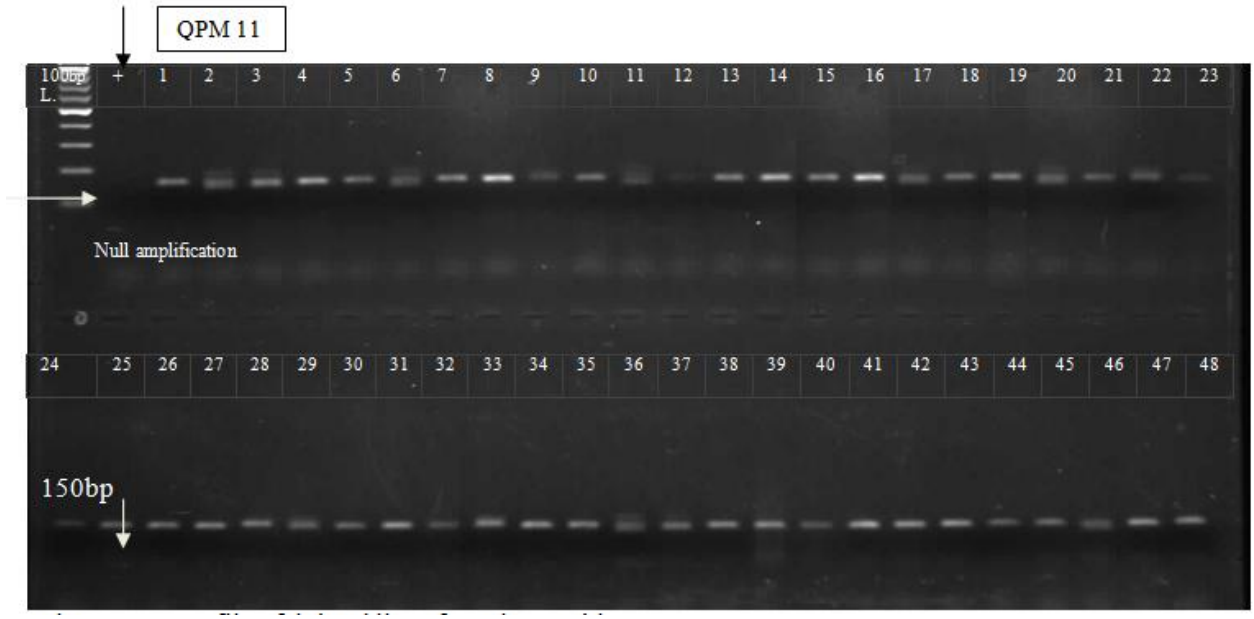

QPM 11
100bp L.
+ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Null amplification
24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
150bp

Fig. 2

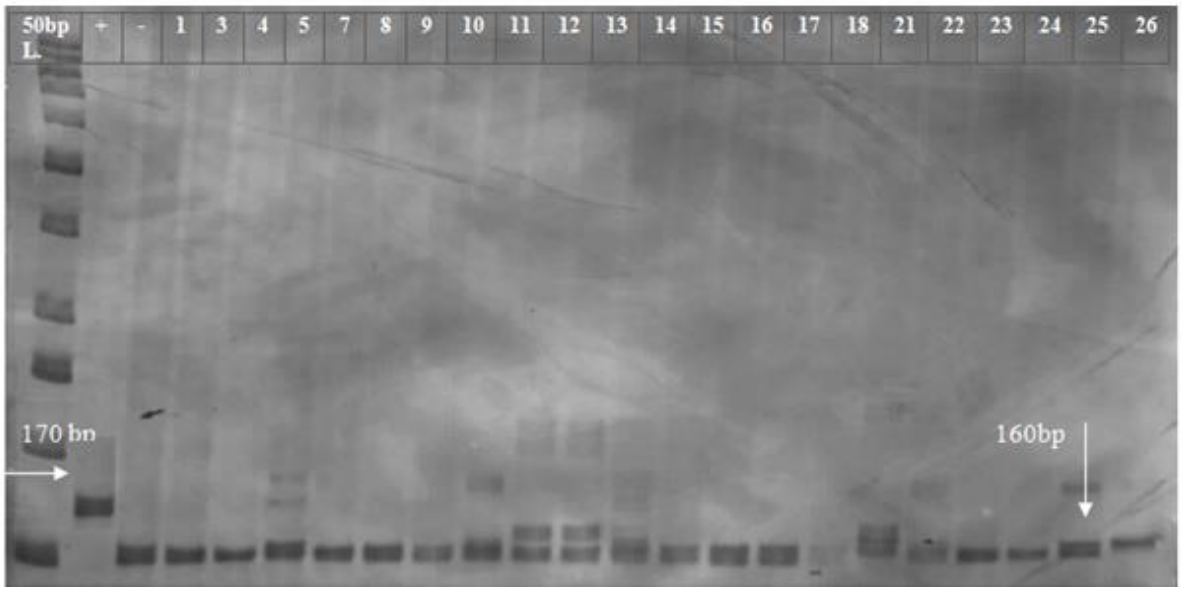

50bp L.
+ - 1 3 4 5 7 8 9 10 11 12 13 14 15 16 17 18 21 22 23 24 25 26
170 bp
160bp

Fig. 3


50bp
40
39
38
37
36
35
34
33
32
31
30
29
28
27
26
25
24
23
22
-
+
170bp
160pb

Fig. 4

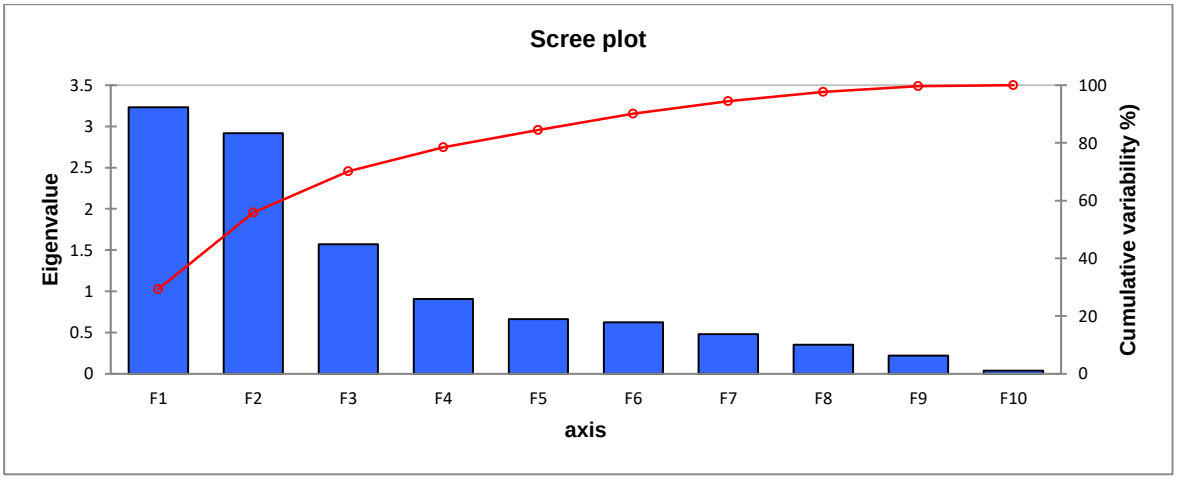
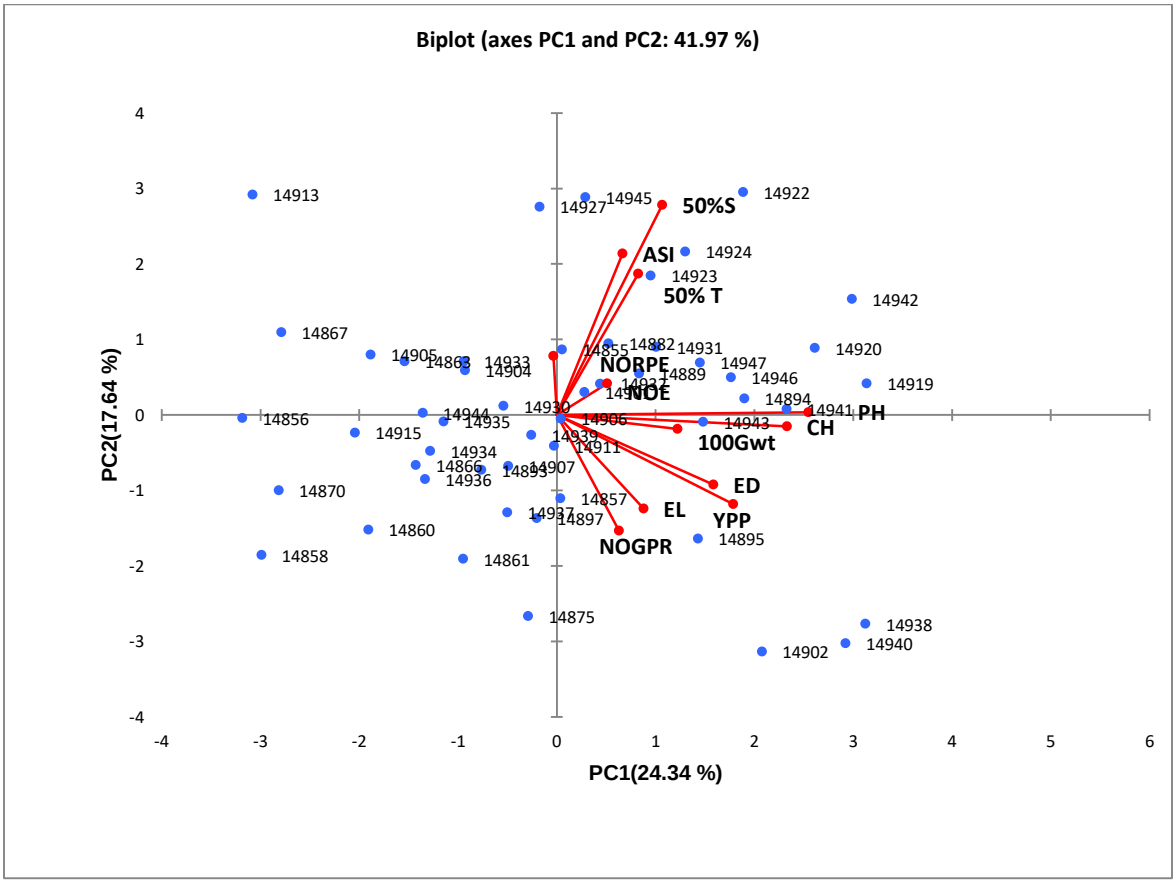

Scree plot
Eigenvalue
3.5
3
2.5
2
1.5
1
0.5
0
100
80
60
40
20
0
Cumulative variability %)
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
axis

Fig. 5


Biplot (axes PC1 and PC2: 41.97 %)
PC2(17.64 %)
PC1(24.34 %)
50%S
ASI
50% T
NORPE
NOE
100Gwt
CH
PH
ED
EL
YPP
NOGPR
14913
14927
14945
14922
14924
14923
14942
14867
14905
14863
14933
14904
14855
14882
14931
14920
14947
14889
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14894
14941
14856
14944
14935
14930
14906
14943
14915
14939
14911
14934
14866
14907
14936
14893
14870
14857
14937
14897
14860
14895
14858
14861
14875
14938
14940
14902

Fig. 6