Selection for Hard Endosperm, Tryptophan Content and Yield Contributing Traits in $F_{3:4}$ QPM Families

Thesis

Submitted to the
Central Agricultural University, Imphal
in partial fulfilment of the requirements
for the award of the degree of

Master of Science (Agriculture)

In

Genetics and Plant Breeding

by

MARIYAPPAN. S.B

CAU/203-A/16(PG)

SCHOOL OF CROP IMPROVEMENT
COLLEGE OF POST-GRADUATE STUDIES
CENTRAL AGRICULTURAL UNIVERSITY (IMPHAL)
Umiam, Pin: 793103, Meghalaya, India

August 2018

Dedicated to

My dear Seniors

and

Juniors

COLLEGE OF POST GRADUATE STUDIES
CENTRAL AGRICULTURAL UNIVERSITY (IMPHAL)
Umiam, Ri Bhoi District
Pin: 793103, Meghalaya, India

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Certified that **Mr. Mariyappan. S.B {**Registration No. **CAU/203-A/16(PG)}** has satisfactorily prosecuted his course of research for a period of not less than two semesters and that the thesis entitled **"Selection for Hard Endosperm, Tryptophan Content and Yield Contributing Traits in $F_{3:4}$ QPM Families"** submitted by him to the Central Agricultural University, Imphal – 795 004 (Manipur) in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Genetics and Plant Breeding** is the result of original research work conducted by him under my supervision and is sufficiently of high standard to warrant its presentation to the examination.

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Date:

(Devyani Sen)
Chairperson
Student's Advisory Committee

Tel: 0364 257 031 (0) / Tel Fax: 0364 257 030
Email: deancpgs@gmail.com

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CENTRAL AGRICULTURAL UNIVERSITY, IMPHAL
Umiam, Ri Bhoi District
Pin: 793103, Meghalaya, India

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This is to certify that the thesis entitled **"Selection for Hard Endosperm, Tryptophan Content and Yield Contributing Traits in $F_{3:4}$ QPM Families"** submitted by **Mr. Mariyappan. S.B [Regn. No. CAU/203-A/16(PG)}** to the Central Agricultural University, Imphal – 795 004 (Manipur) in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Genetics and Plant Breeding** has been approved by the Student's Advisory Committee after oral examination jointly with a Dean's nominee.

(Dwipendra Thakuria)
Professor,
School of Natural Resource Management
Dean's Nominee

(Devyani Sen)
Assistant Professor,
School of Crop Improvement
Chairperson,
Student's Advisory Committee

(Mayank Rai)
Professor,
School of Crop Improvement
Member,
Student's Advisory Committee

Chairman,
School of Crop Improvement

(V.K. Khanna)
Professor,
School of Crop Improvement
Member,
Student's Advisory Committee

Dean of Faculty

(Wricha Tyagi)
Professor,
School of Crop Improvement
Member,
Student's Advisory Committee

Date:

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I hereby declare that the thesis entitled **"SELECTION FOR HARD ENDOSPERM, TRYPTOPHAN CONTENT AND YIELD CONTRIBUTING TRAITS IN $F_{3:4}$ QPM FAMILIES"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other title.

Date: …….. (**Mariyappan. S.B)**

Place: Umiam, Meghalaya Student

ACKNOWLEDGEMENT

This thesis is the result of the contribution of a number of people who deserve special mention. I hereby convey my gratitude and humbly acknowledge their help.

I express my gratitude to ***Dr. Devyani Sen****, Asst. Prof. (Genetics), my major advisor for her direction, valuable suggestion and guidance from the early stage of this research work.*

I convey special acknowledgement to ***Dr. N. B. Singh****, Dean, College of Post-Graduate Studies, Umiam for his vital support. I would like to thank* ***Dr. V. K. Chaudhury****, In-Charge, SCI, College of Post-Graduate Studies, Umiam, Meghalaya for his indispensable help for carrying out this study during my Master's programme.*

I gratefully acknowledge my advisory committee members ***Dr. V. K. Khanna, Dr. Mayank Rai*** *and* ***Dr. Wricha Tyagi*** *and all the faculty and non-teaching staff of the college who have been a source of inspiration and guided me.*

I also owe to my humblest gratitude to Dr. Anup Kumar Mishra, Principle Scientist, NBPGR (Regional Office), Shillong for providing all the facilities, necessary help and his vital support on time during the execution of this thesis work.

I am very much thankful to all my seniors Mr. ***Mayil Samy*** *Mr.* ***Kaleeswaran****, Mr.* ***Jennie,*** *Mr.* ***Dheena Dhayalan,*** *Mr.* ***Sankar Ganesh,*** *Mr.* ***Naveen****, Mr.* ***Pawan****, Mr.* ***Aso****, Ms.* ***Bhakiyalakshmi*** *and Ms.* ***Bharati Lap*** *for their help. Collective and individual acknowledgements are also owed to my friends* ***Girish, Pramod, Gopinath, Akbar, Eben, Irshad, Suraj, Tondonba, Kunguma Selven, Nikhil, Roopesh,, Aren, Sumpi, Chumki, Careen, Flamia*** *and* ***Debjani.*** *I would also like to gratefully acknowledge my beloved juniors* ***Samudhira Pandi, Shanmugam, Krishna, Libi, Nitin, Harsha vardhan, Dhivya*** *and* ***Usha*** *for their timely assistance .*

My parents ***Mr. M. Balamanickam and Mrs. B. Thangamani*** *deserve special mention for their constant support and prayers. No words can describe the gratitude, obligation and thankfulness I owe them. I am also heartily thankful to my brother Mr.* ***S. B. Myleswaran*** *for his sacrifice, blessings, unconditional love and for being the strength behind all my endeavours.*

I also acknowledge the ***Supreme*** *and* ***Universal "Almighty"*** *without whose blessing, I would have never successfully completed this work.*

Place: Umiam *(Mariyappan. S.B)*

Dated: ……..

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LIST OF ABBREVIATIONS/ SYMBOLS

%	Percentage
µl	Micro Litre
^0C	degree Celsius
AAS	Atomic Absorption Spectrometer
ACP	Acid Phosphatase
ADH	Alcohol Dehydrogenase
AMMI	Additive Main effects and Multiplicative Interactions
ANOVA	Analysis of Variance
ASI	Anthesis Silking Interval
BC	Back Cross
CIM	Composite Interval Mapping
CIMMYT	International Wheat and Maize Improvement Centre
Cm	Centimetre
CML	CIMMYT Maize Line
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	Ethylene Diamine Tetra acetic Acid
EST	Esterase
et al.	et alia
$FeCl.6H_2O$	Ferric chloride six hydrated
Fig.	Figure
g	Gram
GCA	General Combining Ability
GCV	Genotypic Covariance
GEI	Genotype X Environment Interaction
GS	Genetic Similarity
H_2SO_4	Sulfuric Acid

h_{bs}	Broad Sense Heritability
Hcl	Hydro Chloric acid
MABB	Marker Assisted Backcross Breeding
MAS	Marker Assisted Selection
MDH	Malate Dehydrogenase
Mg	Milli grams
N	Total number of samples
NaCl	Sodium Chloride
NCD	North Carolina Design
NEHR	North Eastern Hill Region
NIL	Near Isogenic Line
o2	*opaque-2*
OD	Optical Density
OPV	Open Pollinated Variety
OQL	Odisha QPM Lines
PCA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PCV	Phenotypic Covariance
PEM	Protein-Energy Malnutrition
pH	Power of Hydrogen
PIC	Polymorphism Information Content
QI	Quality Index
QPM	Quality Protein Maize
QTL	Quantitative Trait Loci
R	Genetic Gain
R/S	Realized Heritability
R^2	Coefficient of determination
RBD	Randomized Block Design
RIL	Recombinant Inbred Line
RNAi	Ribonucleic Acid Interference
RRS	Reciprocal Recurrent Selection

S	Selection Differential
s	Standard Deviation
SCA	Specific Combining Ability
SDS-PAGE	Sodium Dodecyl Sulfate
SIM	Simple Interval Mapping
SMR	Single Marker Regression
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
TBE	Tris Borate EDTA
TE	Tris EDTA
UMQ	Umiam Maize Cross
UPGMA	Unweighted Pair Group Method with Arithmetic mean
V_e	Environmental variance
V_g	Genotypic variance
viz.	Such as
VMH	Vivek Maize Hybrid
V_p	Phenotypic variance
VQL	Vivek QPM Line
X	Mean
X_b	Base population mean
X_i	Individual lines tryptophan value
X_m	Mean tryptophan value of particular family

ABSTRACT

The present investigation was undertaken for selection of QPM inbred lines with hard endosperm and high tryptophan content in a set of 60 $F_{3:4}$ QPM families shortlisted from four QPM crosses V341 X V 373, CML 165 X V 373, CML 161 X V 341 and CML 161 X V 373. A total of 681 cobs harvested from sib matings within different families were screened for opacity and sixty lines with index scores of 2 and 3 (hard endosperm) and high seed index value was further selected for determining tryptophan content using Hopkins-Cole reaction. The % tryptophan was found to range from a maximum of 0.095% to a minimum of 0.020%. Twenty two lines with tryptophan % in the range of 0.095% to 0.073% could be identified. Differences in tryptophan content between normal and QPM lines were significant. High genetic gain and realized heritability could be recorded for % tryptophan increase in most of these twenty two lines studied. These lines were further charcterized at the DNA level along with dive low QPM and two normal maize genotypes using twenty *o2* specific reported SSR markers. Distinct polymorphism was observed between the normal and a majority QPM inbred lines with respect to all the three gene specific SSR markers *phi 057*, *phi 112* and *umc 1066* validating the identity of the lines. Cluster analysis using Unweighted Neighbour Joining based on allelic data grouped the lines into three major clusters. QPM lines with high % tryptophan grouped into Clusters I and II while QPM lines with low % tryptophan grouped into Cluster III. The genetic distance based on dissimilarity matrix ranged from a minimum of 0.10 to a maximum of 0. 47. The polymorphism information content (PIC) ranged from 0.19 to 0.65 with an average of 0.44. Yield/plant calculated for the different families as total kernel weight (g) was the most variable traits and ranged from 88 to 28 g for the twenty two high QPM families. Based on these studies, QPM lines with more than 0.090% tryptophan can be shortlisted for further use to initiate a hybrid breeding programme for QPM.

Keywords: Quality Protein Maize (QPM), light box index, tryptophan estimation, *o2* gene.

Chapter 1

Introduction

Maize (Zea mays L., 2n=20) is a highly cross pollinated monocot crop of family Poaceae, known as “Queen of Cereals” and is second most important cereal crop in the world in terms of acreage, contributing 2 % in global production. In India, maize ranks third after rice & wheat and contributes 9 % of total grain production. As a primary cereal crop it occupies an important position in world economy because of its diverse functionality. Maize in East and Southeast Asia is primarily used for animal feed while it is the staple diet in Africa, Central America and South Asia. In India maize accounts for 89 % domestic consumption and the 10 % share in total food grain production with a 7 % consumption growth rate because of growing demand for poultry feed and industrial use. This has encouraged more farmers to bring larger area under cultivation in both *kharif* (73 %) and *rabi* (27 %) seasons (Anonymous, 2018). Maize cultivation in North East India is second in importance to rice where it is primarily used as livestock feed.

General uses of maize in India varies from poultry and cattle feed to starch and brewery, human consumption besides serving as raw material for several agro-allied industries indicating that the maize value chain for both backward and forward linkages have evolved substantially. By 2020, the world will have a population of approximately 7.7 billion people which is projected to rise to approximately 9.3 billion by 2050. The demand for maize in the developing world will be doubled as a result. Normal maize however with high levels of α- zein protein is essentially devoid of essential amino acids tryptophan and lysine from the major prolamin fraction of the endosperm leading to poor nutritional quality.

Therefore, overdependence on maize-based diet without complementary food sources may lead to nutritional deficiency-related diseases. To alleviate this problem, researchers attempted to create nutritionally improved maize stimulated by the discovery of Howard Jones in 1935 and Singleton in 1939 who reported of naturally occurring recessive maize mutant with 30 % more lysine and 55 % more tryptophan compared to normal maize (Prasanna *et al.*, 2001). The implications of this discovery was considered remarkable where it was found that recessive *o2* mutation in the homozygous state improved the lysine and tryptophan content in maize, while leaving the quantity of protein unchanged (Vasal, 2000; Vivek *et al.*, 2008).

The discovery of maize with improved protein quality was however associated with several undesirable agronomic characteristics like reduced yield, chalky endosperm and susceptibility to ear rot disease and storage pests. Therefore, a research development breeding programme via modified backcross and recurrent selection was initiated by CIMMYT (International Wheat and Maize Improvement Centre, Mexico), for incorporation of recessive *o2* genes for higher lysine and tryptophan content along with polygenic modifier genes for hard endosperm which came to be known as Quality Protein Maize or QPM (Vasal, 2000).

QPM development programmes subsequently have focused on converting elite non-QPM inbred lines and Open Pollinated Varieties (OPV) to elite QPM inbred lines using backcross or pedigree breeding methods. These breeding programmes followed two unique and essential steps for development of QPM. Firstly, the identification of *o2* allele in homozygous recessive condition with a hard endosperm followed by confirmation of QPM quality through biochemical analysis of percent tryptophan and protein in the converted maize sample. In conventional approach of development of QPM, light box screening has been practiced to differentiate hard endosperm QPM lines by visually grading the endosperm opacity (Vivek *et al.*, 2008).

QPM hybrids are advantageous over normal maize in terms of yield potential comparable to the best normal hybrids, assured seed purity, uniform and stable endosperm modification and superior protein quality. Where conventional approaches are time consuming, use of Simple Sequence Repeats (SSR) for *o2* and *o2* modifiers help to accelerate the pace of QPM conversion programs through Marker-Assisted Selection (MAS) and genetic diversity studies (Babu *et al.*, 2005). MAS using Polymerase Chain Reaction (PCR) based markers such as SSR is an attractive option as it helps to easily detect both the simple inherited *o2* and associated polygenic modifier genes in the newly developed cultivars with high yield potential (Jompuk *et al.*, 2011). Genetic analysis of QPM has shown that the *o2* and modifier genes that significantly influence the protein quality and endosperm modification is found to be tightly linked to chromosome 7 (Babu *et al.*, 2005). Therefore, selection of QPM inbred lines with *o2* specific and modifier genes will be easier using *o2* specific SSR markers located on chromosome 7. Modifiers located on chromosomal bins 1.01, 1.08, 2.07, 3.04 and 9.05 have also been reported to be associated with vitreous endosperm and increased tryptophan and lysine content by association with *o2* gene (Krishna *et al.*, 2012).

Despite its nutritional superiority, QPM cultivation is yet to gain wider acceptance because hybrids need to be produced with similar agronomic performance

as normal hybrids while retaining enhanced nutritional quality. In North Eastern Hill Region (NEHR) of India, soils are predominantly acidic and it is imperative that QPM hybrids tolerant to acidic soil conditions are developed along with better yield. If successful, it would provide a cost effective access to high quality protein at low cost.

A combining ability study using half diallel mating involving crosses CML 161 X CML 165, CML 161 X V 390, CML 161 X V 341, CML 161 X V 373, CML 165 X V 341, CML 165 X V 373, V 390 X V 341 and V 341 X V 373 was initiated in *kharif* 2015 at College of Post Graduate Studies, Umiam (Backiyalakshmi, 2016). It was found that under acidic soil conditions, parental line CML 165 was a good general combiner. Among the crosses, CML 165 x V 341 showed promise for both tryptophan content and grain yield. Although there are no absolute values that define QPM, the acceptable standards for tryptophan should be higher than 0.070 % and therefore recycling of the segregating generations of the F_1 hybrids was initiated to identify superior lines with superior tryptophan content. The six F_2 populations were sib mated within each population and segregants with kernel modification score 3 and high seed index were advanced to the F3 generation (Khati, 2018). This was followed by another round of sib mating within families followed to raise the $F_{3:4}$ generation.

The current study focuses on identification of QPM lines from the $F_{3:4}$ families to be characterized for high percent tryptophan values with the following objectives:-

1. Study of opacity of different inbred lines by light box screening.
2. Estimation of tryptophan amount for different sib mated inbred lines.
3. To select for homozygous recessive *o2* genes at seedling stage of $F_{3:4}$ families using reported SSR markers for *o2* and *o2* modifiers.

Chapter 2

Review of Literature

The nutritional significance of maize endosperm protein was genetically enhanced in Quality Protein Maize (QPM) with increased lysine and tryptophan content interchangeable with normal maize in yield and kernel phenotype. The increase in tryptophan is directly correlated with the increased level of the γ-zein proteins in maize which leads to higher niacin synthesis. Development of QPM involved two essential steps. The first step involved identification of segregants in a family or population of maize with *o2* (*opaque-2*) alleles in homozygous recessive condition. The second step involved selection of lines with kernel modification to resemble normal maize along with biochemical confirmation for presence of essential amino acids within prescribed QPM standards (Vivek *et al*., 2008). The spontaneous mutant *o2* gene was associated with soft endosperm, dull kernel appearance, brittleness and susceptibility to disease and stored pests and therefore systematic introgression of kernel modifiers for endosperm hardness and amino acid responsible for enhancing tryptophan and lysine content were required to be incorporated. The combined use of the two genetic systems involving the *o2* gene and genetic modifiers of *o2* locus found to be located on chromosome 7 for high tryptophan and lysine with the phenotype and yield of normal maize, has led to the development of competitive QPM genotypes of today (Vasal, 2000; Prasanna *et al*., 2001; Bantte and Prasanna, 2003; Holding *et al*., 2008; Babu and Prasanna, 2014; Babu *et al*., 2015).

The development of nutritionally superior maize protein was initiated by CIMMYT in the mid-1960s and several plant breeders and institutions have contributed to the development of QPM. Vasal (2000) reviewed the quality protein maize story with respect to the breeding strategies at CIMMYT (International Wheat and Maize Improvement Centre, Mexico) involving introgression of the *o2* gene and the genetic modifiers involved in correcting the chalky endosperm defects of the *o2* locus in varied genetic backgrounds leading to breeding agronomically superior QPM. Prasanna *et al*. (2001) reviewed the critical genetic, biochemical and molecular basis features for development of market competitive QPM since adverse pleiotropic effects of high lysine mutants restricted the use of mutants in their original form. The review highlights various multidisciplinary approaches adopted by CIMMYT to develop QPM hybrids with

increased levels of γ-zein proteins leading to kernel modification to acceptable agronomical standards.

Work on several aspects involving QPM has been done since, and the following review focuses on the QPM breeding with respect to nutritional significance, with respect to breeding for yield and yield related traits and genetic variability at the biochemical and molecular level for QPM genotypes governed by *o2* and *o2* modifier genes.

2.1 Nutritional significance of Quality Protein Maize:

Biofortification has been found to be effective in cultivated species with sufficient genetic potential to increase the essential elements lacking in human diet through breeding programme. Intake of biofortified food involves increase in the concentration of available nutrients through development of crops with increased ability to acquire essential elements (White and Broadley, 2009).

QPM, besides containing 55 % more tryptophan compared to normal maize also is also of increased biological value which is the amount of absorbed nitrogen required to provide necessary amino acids for various metabolic activities. While biological value of normal maize is 45 % that of *o2* maize is 80 % and a minimum of 125 g of daily intake of *o2* maize can help equilibrate nitrogen content with increased nitrogen balance index. Other nutritional significance include lower leucine and higher carotene content, higher calcium and carbohydrate utilization (Prasanna, 2001).

Vivek *et al*. (2008) discussed the effect of QPM with enriched lysine and tryptophan content used for feeding trials in pigs compared to pigs fed solely with normal maize. Pigs fed with QPM, showed increased in body weight, maintenance of amino acid balance. This has made a sizeable impact in economic upliftment of small scale farmers the world over involved in pig rearing. They also discussed the impact on human nutrition in Ghana and Columbia where the staple diet is maize. In both studies, incorporation of QPM in the diet of children showed reduced malnutrition effects of kwashiorkor with growth enhancing capabilities.

Nuss and Tanumihardjo, (2011) reported that compared to normal maize, QPM has twice the amount of lysine and tryptophan, as well as protein bioavailability that rivals milk casein. It was found that 100 g QPM is required for children in Africa to maintain adequacy of lysine, the most limiting amino acid, and nearly 500 g is required for adults. This represents a 40 % reduction in maize intake relative to common maize to meet protein requirements. A biofortification with QPM

would provide the estimated average requirement of vitamin A to children who consume 200 g/d dry maize as part of their staple diet.

QPM feeding trials in multi-location showed that improved nutrition and also resulted in the improvement in height and weight of children particularly in Southern Ethiopia where whole population solely rely on maize. In addition to that diversified use of QPM led to use as livestock feed source with improved biological value (Shiferaw *et al*., 2011).

Increased lysine and tryptophan content in QPM increases the biological value to double the amount in normal maize protein it would drastically reduce the amount of other protein supplements. Aditionally, the content of amino acids such as histidine, arginine, aspartic acid and glycine are also enhanced in QPM with a simultaneous decrease in glutamic acid, alanine and leucine. Decrease in leucine makes leucine–isoleucine ratio more balanced, and helps to provide more tryptophan for niacin biosynthesis for combating pellagra (Babu and Prasanna, 2014).

2.2 Yield and yield contributing characters:

A study was conducted to compare grain yield, endosperm modification and protein quality traits by Pixley and Bjarnason (2002) in a set of eighteen single-cross, eighteen three-way, eighteen double-cross hybrids of QPM and eight open pollinated cultivars of QPM evaluated at 13 tropical locations in which hybrids recorded 13 % higher grain yield and 2 % higher protein concentration than the normal open pollinated maize cultivars. Additive Main effects and Multiplicative Interactions (AMMI) analysis indicated that GEI effects on yield and yield contributing characters and endosperm modification were considerably higher for QPM hybrids than normal open pollinated maize cultivars.

Ravikant *et al*. (2006) studied gene effects of metric traits like yield and yield components in six population *viz.* two parents, F_1, F_2, BC_1 and BC_2 which were generated from a set of ten QPM inbred lines of CIMMYT origin. Study on morphological and yield and yield contributing traits revealed that additive gene effects was predominant followed by epistatic effects for shelling %, kernel vitreousness and yield and duplicate type of epistasis was observed for grain yield per plant, kernel specific gravity, shelling % and kernel vitreousness. So for these additive gene effects, recurrent selection for General Combining Ability (GCA) and for non additive gene effects, recurrent selection for Specific Combining Ability (SCA) was proposed to be effective.

Scott *et al*. (2009) studied about combining ability for amino acid content and kernel opacity in temperate QPM inbred lines derived from crosses between two CIMMYT QPM (donor lines) inbred lines and from Iowa State University (recurrent parent). They made reciprocal as well as direct crosses using NCD II and diallel mating design for evaluating agronomic traits where specific combining ability was found to be significant for grain yield and general combining ability was significant only for male parents which could described that additive and non-additive gene effects were playing a major role in determining yield. Also they found that higher yield in hybrids from various QPM donor lines compared to hybrids from similar donor lines which implied major role of QPM lines with good combining ability.

Mbuya *et al.* (2010) performed participatory selection and characterization of QPM varieties in Savanna agro ecological region of DR-Congo in which QPM varieties were planted with and without chemical fertilization and selected based on agronomic traits such as days to maturity, plant and ear traits like plant height, ear height, yield and yield attributing components like number of rows per ear, number of kernel per row and seed index. There was significant differences among QPM varieties under both the treatments for several agronomic traits *viz.* grain yield, days to flowering, plant height and plant and cob characters which revealed better performance of QPM under normal and stress conditions.

Shrestha, (2013) carried out yield stability analysis of QPM genotypes for GEI in Terai region of Nepal in which they observed considerable variation among genotypes and environments for grain yield which showed that mean squares of environments were highly significant and genotypes and GEI were non-significant which could led to identify promising QPM genotypes with high mean grain yield and better adaptability for general cultivation in Terai region of Nepal.

Singh *et al.* (2014) studied about growth and yield losses difference in a set of QPM and normal maize genotypes to variable levels of ozone in which they noted that Analysis of Variance (ANOVA) results showed significant interaction between age x treatment in both cultivars. In case of low dose of ozone, plants showed hermetic effects, foliar injury and also increase in anthocyanin pigmentation was led to damage reproductive structure. Through magnitude of response to ozone in normal maize lines and QPM for different ozone levels showed that better performance of QPM than normal maize

Lal and Singh, (2014) assessed the magnitude of genetic variability for morphological and quality traits in a set of fifty seven QPM genotypes by an experiment

wherein heritability and genetic advance were found to be moderate for protein, tryptophan, lysine concentration as well as days to 50 % silking, number of rows per ear and grains per row indicating predominance of non additive gene action. This notable action of non additive gene action ensured that environment plays a major role and problem was associated with improvement of these characters, but results of heterosis breeding led to be effective for development of non additive gene action.

Ofori *et al*. (2015) studied performance of six extra-early QPM inbred lines to assess the hybrids and their parents for their agronomic performance using a partial diallel cross design. ANOVA for six parents along with their fifteen hybrids revealed significant environmental effect for all the traits studied and they observed high values of relative heterosis for grain yield in various crosses involving different combinations of the six inbred lines and suitability of various parents for different yield and yield contributing characters. They suggested that these parents could be used in hybridization programmes as donors for their superior agronomic traits.

Annor and Badu-Apraku (2016) studied twenty four extra-early QPM inbred lines under stress and non-stress conditions about various gene actions controlling grain yield and other agronomic traits in which selected inbred lines were crossed based on North Carolina Design II (NCD II) with four checks. These lines were studied to evaluate the performance under drought and low nitrogen stress and non-stress environments and they found that some of the inbred lines possessed favourable alleles for stress tolerance which could be used for population improvement and development of stress tolerant and high yielding hybrids.

2.3 Biochemical and molecular divergence studies for endosperm modification:

Huang *et al.* (2005) studied about the α-zein proteins reduction in transgenic lines containing the coding region of 19-kDa α-zein gene and the high free lysine line, M27908 consist a transgene with the coding region of CordapA which was insensitive to lysine inhibition. Analyses on six different F_1 genotypes for total amino acid analysis revealed that transgenic lines have elevated lysine levels when compared to control and further free amino acid and lysine metabolite analysis of F_1 seeds showed that correlation between the transgenic zein reduction and the enhanced lysine biosynthesis by CordapA expression

Olakojo *et al.* (2007) assessed the level of lysine and tryptophan in six QPM varieties and evaluated them at various agro-ecological zones for yield performance in which they found that improvement in lysine content positively

correlated with zein protein and tryptophan was positively correlated with crude protein. Through this study they identified three QPM varieties with improved tryptophan and lysine content which could perform consistently in different locations and could be suggested for commercial use.

Gutierrez-Rojas *et al.* (2008) carried out study on phenotypic characterization of QPM for endosperm modification and amino acid content in a segregating recombinant inbred population in which they evaluated in 146 Recombinant Inbred Lines (RILs) obtained from the cross between the *o2* inbred B73*o2* and the QPM inbred CML161. In maize kernel lysine and tryptophan amount, methionine, opacity, vitreousness and endosperm texture were found to be highly correlated with each other and endosperm texture and amino acid contents were found to be negatively correlated.

Comparably, QPM lines with improved tryptophan content (0.05 to 0.11 %) than normal maize (0.02 to 0.05 %) and based on glyoxalic acid method, samples with values greater than 0.07 % for tryptophan and greater than 0.7 % Quality Index (QI) can be consider as QPM. In QPM positive correlation between tryptophan and lysine content improvement than the other non zein proteins and lysine amount is four times of tryptophan amount found to be notable discovery and this could be helpful to reduce the time and cost of estimation of both the amino acids (Vivek *et al*., 2008).

Hossain *et al.* (2008) evaluated and identified promising QPM inbred lines and experimental hybrids with respect to grain yield as well as endosperm protein quality over two locations. Studies on endosperm modification, crown opaqueness, ear appearance and combining ability showed significant differences among QPM genotypes and segregation of several kernel modifier genes with different dosage effects led to effect on endosperm vitreousness showed significant differences among genotypes.

Hasjim *et al.* (2009) studied action of *o2* mutation and QPM on kernel composition, starch structure, property and enzyme digestibility in five maize inbred lines including QPM by nitrogen estimation (Micro-Kjeldahl method), tryptophan estimation (Nurit *et al*., 2009) and starch content analysis (Megazyme total starch assay kit following AACC Method). These results showed that *o2* mutants contained less protein than their wild type counterpart and reduction in the starch content result from early termination of the starch biosynthesis in the *o2* mutant followed by faster hydrolysis by α-amylases than dry-ground wild type maize to provide energy for animal growth and to produce glucose for alcohol fermentation.

Nurit *et al.* (2009) developed an tryptophan estimation method with higher precision (r^2=0.95 between technical replicates) than acetic acid method (r^2=0.82 between technical replicates) in which tryptophan reacts with glyoxylic acid (0.1 M) in the presence of sulphuric acid (7N) and ferric chloride (1.8 Mm), producing a colored compound that absorbs at 560 nm and results suggested that samples as QPM which are with tryptophan content values higher than 0.07 % which could be validated by comparison of data with acetic acid-based colorimetric procedure and high pressure liquid chromatography.

Sofi *et al*. (2009) worked on the mechanism of endosperm modifiers functioning and observed that presence of modifier genes has negative correlation with α-zeins and positive correlation with γ-zeins in *o2* backgrounds which helps to initiate formation of γ-zeins proteins with increasing hardness to a particular level as endosperm modification in maize. Those modification in endosperm would possibly noted by light box screening as conventional method in which score 1 and score 5 would imply fully modified kernels as extreme hardness and extreme softness respectively, so for breeders a scale of 2 and 3 would be recommended in early breeding generations for average kernel modification with improved tryptophan and lysine content.

Ignjatovic-Micic *et al*. (2010) studied selection for kernel modification and tryptophan analysis in segregating generations of a different cross combination between QPM, *o2* maize and standard lines. Analyses on kernel modification revealed that more than 60 % of all three types of crosses and crosses between QPM and *o2* lines enriched with higher tryptophan amount compared to cross between standard lines and QPM which could sufficiently described that *o2* genes had significantly higher tryptophan content and better kernel modification.

Wu *et al*. (2010) studied about zein proteins in maize endosperm and action of γ-zeins on endosperm modification in which high level accumulation of zein protein leads to devoid of tryptophan and lysine in maize endosperm which results in poor grain-protein quality. They reported that 27 and 16-kDa γ-zeins were knocked-down by γRNAi (knock-down lines or natural null mutants with γ-zein genes) which resulted in partial. This study showed that *Mo2*/+ and *o2*/*o2 genotypes* with enhanced accumulation of the 27-kDa γ-zein and normal levels of the other zeins and RNAi/+ genotypes showed similar phenotype to *Mo2/+* and *o2/o2* with reduced expression of the 27 and 16-kDa γ-zeins which revealed that abnormalities in γ-zeins protein body structure and starch granules due to RNA interference (RNAi) leads to affect endosperm modification (Liu *et al*., 2016).

Wu *et al*. (2011) studied about action of γ-zeins on endosperm modification by F_1 hybrid of a cross between B73 x Mo17 where RNAi technology employed for α zeins which reduce 22 and 19-kDa α-zeins in six QPM kernels compared to normal maize kernels. Half of the kernels from (B73 x Mo17) x P6z1RNAi/+ were opaque by the action of *Mo2* genes to overcame RNAi, which can be used in backcross to transfer the trait into normal maize. Further molecular diversity studies of this QTL (Quantitative Trait Loci) on chromosome 7 was found to be very useful for selection of QPM inbred lines with *o2* and *o2* modifiers and suggested that γ-zeins are essential for endosperm modification to restore protein body density in QPM.

Holding *et al.* (2011) studied Characterization of *o2* modifier QTL in a panel of three hundred and fourteen recombinant lines which were derived from a cross between K0326Y QPM and W64A*o2* followed by modified single seed descent method. From S_6 generation for each RIL, zein protein composition analysed where finely ground endosperm was mixed and vortexed with zein extraction buffer and proteins from 1 mg of endosperm flour were resolved by 12 % SDS–PAGE (Sodium Dodecyl Sulfate-Poly Agarose Gel Electrophoresis) (Wallace *et al.,* 1990) to verify a kernel hardness selection with homozygous *o2* genotype by light box screening method (Vivek *et al*., 2008). Genetic linkage analysis of the RIL population confirmed that three of the previously identified QTLs associated with *o2* endosperm modification was found to be greater in K0326Y QPM which is associated with 27-kDa γ-zein gene (Holding *et al*., 2011; Liu *et al*., 2016).

QPM with increased tryptophan, lysine content and hard endosperm primarily developed for utilization in tropical and sub-tropical regions. Ignjatovic-Micic *et al*. (2013) studied about development of QPM adapted to temperate regions by a set of seventy two hybrids, derived from 27 QPM and three MRI standard lines. Initially kernel modification scores were given by light box screening method, then selected lines forwarded for tryptophan analysis where flour hydrolysate obtained by overnight digestion with papain solution at 65^0C reacted with 2 ml of reagent containing 270 mg of $FeCl_3.6H_2O$ dissolved in 1 litre of glacial acetic acid and 2 ml of 30 N H_2SO_4 and finally incubation at 65°C for 15 minutes followed by absorbance was read at 560 nm (Vivek *et al.,* 2008) in which tryptophan content ranged from 0.046 to 0.088 % and they identified five hybrids QPM6 x L1, QPM10 x L2 , QPM24x L2 , QPM17 x L1 and QPM18 x L1 with high tryptophan content (0.071 to 0.081 %), yield at the level of standard hybrids (96 to 114 %) and % of good kernel modifications (type 1 and 2) was over 70 % in all hybrids which were suggested to be highly adapted in temperate regions.

Salazar-Salas *et al*. (2014) conducted an experiment for biochemical characterization of QTLs associated with endosperm modification in QPM using RILs with three identified trait loci in bins 1.06, 7.02 and 9.03 associated with endosperm modification. Effects of these QTLs on protein accumulation and starch properties showed that expression of QTL in bin 7.02 leads to greater accumulation of γ-zein proteins in vitreous kernels and also QTL in bin 9.03 is responsible for vitreous kernel character. They suggested that these QTLs associated with kernel modification and selection of kernels based on this association could be effectively used in plant breeding programme.

Stamp *et al.* (2014) studied introgression of waxy *o2* genes into waxy maize landraces of ethnic minorities in North Vietnam where two waxy landraces were crossed with QPM lines of modern genetic background, so four crosses were generated from the best performing top crosses and tested with commercial Thai waxy hybrids. Thus results showed that harvested ear waxy hybrids were had similar results with check in grain protein, tryptophan and highest amount of yield which indicated successful introgression of desirable traits for quality improvement.

Hussain *et al*. (2015) studied effects of additive and non additive gene action on protein quality traits *viz.* protein, tryptophan and lysine content in a set of QPM lines and hybrids under normal and drought conditions using a complete 8 × 8 diallel mating design. This study revealed that inheritance of major quality traits like lysine content in maize and protein quality was under the control of additive effects with partial-dominance in both normal as well as in drought stress conditions.

Liu *et al*. (2016) studied about QTL at the 27-kDa γ-zein locus which confers enhanced expression of quality protein and leads to endosperm modification on direct and reciprocal crosses of QPM line K0326Y QPM (with *qγ27*) and B73 (without *qγ27*). Due to triploid nature of endosperm, they found three, two, one and zero dosage of QTL *qγ27* in K0326Y QPM, K0326Y QPM × B73, B73 × K0326Y QPM and B73 respectively and phenotypic analysis revealed that protein level of 27-kDa γ-zein was positively correlated with the genetic dosage of *qγ27* in QPM. They identified QTL affecting expression of 27-kDa γ-zein (*qγ27*) and mapped on the same region as the major *o2* modifier on chromosome 7 and they found that gene structure of *qγ27* was unstable which leads to frequent endosperm modification in QPM.

2.4 Molecular divergence studies for *o2* and *o2* modifier genes:

Genetic diversity analysis for fifty seven maize lines which including inbred lines and open pollinated lines from CIMMYT were performed by Warburton *et*

al. (2002) in which all the selected lines for this study forwarded for molecular studies. A total of 104 SSR primers were used to analyse polymorphism and genetic relationship among these lines by dendrogram and dissimilarity matrix. In this study they found that some lines from same population showed more variation compared to lines from different population by cluster analysis which revealed that positive and negative correlation between those lines pedigree data and cluster analysis of these selected SSR primers and high level of genetic diversity among the selected lines.

Abundance of allelic variation on maize genome led to identify hundreds of mapped SSR loci which are PCR-based, co dominant, locus-specific, highly reproducible, hyper variable, informative and reasonably easy to use in genotype characterization, genome analysis and gene mapping and these SSR profiling results expected to effectively complement the morphological characters which helped to differentiate the genotypes. Bantte and Prasanna (2003) studied about genetic polymorphisms in twenty three QPM inbred by 43 SSR markers in which Polymorphic Information Content (PIC) values ranged from 0.08 (*phi057*) to 0.80 (*bnlg439*) where results showed that among 43 SSR markers, 36 found to be polymorphic and 7 markers were monomorphic and only *phi057* revealed the presence of *o2* gene in all the QPM inbreds under study and which used to distinguish normal maize and *o2* maize. Cluster analysis and Genetic Similarity (GS) matrices for these 23 QPM lines showed that high levels of heterozygosity in majority of the Indian QPM lines and in one CIMMYT QPM inbred, CML188 which revealed the difference between Indian QPM and CIMMYT QPM lines.

Duarte *et al*. (2004) evaluated protein contents in QPM hybrids obtained by conversion of three normal elite lines through a series of modified backcrosses. A total of twenty five traits which include thirteen agronomic and twelve grain traits were evaluated by *o2* specific SSR markers. Analyses on QPM and normal maize revealed that QPM hybrids obtained by conversion with increased tryptophan and lysine content compared to normal maize and also those hybrids had similar performance compared to original version for most of the agronomic traits including grain yield. Genetic diversity studies revealed that differences in protein composition and kernel modification between the normal and QPM versions of the converted hybrids.

Xia *et al*. (2004) studied genetic diversity for one hundred and fifty five CIMMYT inbred lines which including white and yellow kernels from lowland tropical region. These lines were analysed with 79 SSRs primers for polymorphism and diversity presence within and between the populations. In diversity studies, number alleles could be found used to correlate with the number of samples used and in this

study they reported more number of alleles than the number of samples used by the use of SSR primers which were span around the maize genome used and cluster analysis on these lines showed mixed origin of population which could be forwarded for Reciprocal Recurrent Selection (RRS) to choose genetically distant CIMMYT lines for breeding programme.

Babu *et al.* (2005) reported a rapid conversion of normal maize lines to QPM by marker-based backcross breeding programme in which incorporation of the *o2* gene followed by confirmation with phenotypic selection for kernel modification was practiced. MAS was performed with *o2* specific SSR markers (*phi057, phi112* and *umc1066)* for recovering maximum recurrent parent genome with the *o2* gene. Besides phenotypic selection for agronomic traits such as days to 50 % silking, resistance to *Turcicum* and *Maydis* blight, pollen shedding ability and seed yield also focussed for effective selection. Among these 3 markers, *phi112* exhibited dominant relationship and *phi057, umc1066* exhibit codominant relationship which was suggested for development of QPM breeding programmes and they identified that QPM version of normal inbred lines with 9-12 % increase in grain yield 0.72 % increase in endosperm protein.

Jompuk *et al*. (2006) used *o2* specific SSR markers *phi057* and *phi112* for early detection of tryptophan content in maize endosperm of three popcorn populations of S_0 generation where QPM and non-QPM lines were analysed with *phi057* and *phi112* markers for protein and tryptophan content to differentiate heterozygous and homozygous populations. Their studies revealed that *phi057* which is codominant marker could be used as an effective tool and it is more feasible than dominant marker *phi112* for early indication of all three QPM genotypes, *O2O2*, *O2o2* and *o2o2* at seedling stage.

For biochemical and molecular characterization, nineteen QPM inbreds were selected from thirteen elite QPM lines based on agronomic performance and tryptophan content. PIC values (0.06-0.70) and GS matrices implied that wide variation among of selected 50 SSR markers and they identified that VQL 2 and VQL 8 can ideally be used as parents for mapping the amino acid modifiers for tryptophan content where both were differ significantly for tryptophan content (0.51 % and 0.94 % respectively) and they reported that these sister lines are ideal to map amino acid modifiers (Babu *et al*., 2009).

Variability analysis of normal maize and *o2* inbred lines for good agronomical character and tryptophan analysis was done by Ignjatovic *et al*. (2009)

where genetic variability studies with *o2* specific primers, primers for amino acid modifiers and primer for endosperm hardness modifier gene showed difference between normal and *o2* inbred lines and also implied that QPM lines with higher tryptophan but poor agronomic performance would be poor and some of the lines with low tryptophan values but good agronomic performance. They have concluded that genotypes which were selected based on genetic diversity studies and phenotypic selection with varies tryptophan content should be forwarded for phytopathogenicity test in which it could be tested in field plots to complement them with agronomical characteristics.

Jompuk *et al*. (2011) studied about to conversion of normal inbred lines to QPM inbred lines by the backcross method and MAS of the *o2* gene where crosses were made between normal maize inbred lines (Agron 20 and Agron 29) and QPM lines (Pop65C6-46 and Pop65C6- 55). In BC_1S_2, seven homozygous recessive (*o2o2*) plants identified by using *phi057* which were considered as converted QPM lines and forwarded for tryptophan estimation. Estimation of tryptophan revealed that it ranged from 0.70 to 0.84 % which was in the range known for *o2* maize (0.80) and they had a similar content of overall protein compared with normal maize (7.35 to 7.72 %) but they were similar with normal maize compared to yield and other agronomical characteristics.

Babu *et al.* (2012) studied genetic diversity of QPM and normal inbred lines from North Western Himalayan region, other parts of India and CIMMYT for *o2* genes by SSR markers to identify highly polymorphic SSR loci which are best suited for diversity and mapping studies. A set of forty eight maize inbred lines comprised of QPM and normal maize used in this study and out of 75 SSR markers 97 % was found to be polymorphic loci and *mmc0371*, *umc2364, umc1568*, *bnlg1600*, *phi026*, *umc*2071 and *bnlg1904* markers are reported with high polymorphism which could be effectively used in molecular breeding programmes, QTL mapping studies and genotype pairs suggested that VQL 2 and CML 173 could be ideal parents for mapping modifiers.

Krishna *et al*. (2012) studied genetic diversity of sixty three QPM inbred lines which were developed from India and Mexico by using SSR markers. PIC and cluster analysis revealed that out of 48 SSR markers, 11 markers were polymorphic and these data used to generate dendrogram for selected genotypes which classified the whole population into two major clusters with GS of 66 to 97 % to measure genetic similarity coefficient between the selected inbred lines which could be used to study about pedigree relatedness. These results showed that CML142 (w) could be

suggested as a better combiner with most of the selected QPM lines for the development of hybrids suitable for India.

Gupta *et al*. (2013) studied about development of improved version of normal Vivek Maize Hybrid 9 (VMH 9) in which parental lines (CM 212 x CM 145) taken for introgression of *o2* genes by using as donor stocks in two different crosses (CM212 X CM180 and CM145 X CM 170) and genotypes with greater than 90 % recovery of the recurrent parent genome were selected in BC_2F_2 which could be forwarded for seed multiplication. In this study they recovered Vivek QPM 9 with 94 % recovery of recipient parent genome coupled with 41 % increase in tryptophan, 30 % increase in lysine content and increased grain yield than Vivek Sankul Makka 11 and VMH 9 and disease resistance characteristics of the original hybrid Vivek Maize Hybrid 9 which was adopted in several hill states of North Western and North Eastern Himalayan regions.

Igniatovic-Micic *et al*. (2013) studied F_2 population of crosses between QPM lines developed for temperate regions and commercial lines with standard kernel quality. The crosses were tested for grain yield, moisture at the harvest period, tryptophan, protein contents and QI with high grain yields for further studies where five hybrids with high tryptophan content (0.071 to 0.081 %), QI ranged from 0.71 to 0.74 and high grain yield at par with standard hybrids were identified. These results could be attributed the action of modifier genes which responsible for vitreous endosperm and resistance to storage pests.

Two *o2* specific Near Isogenic Lines (NILs) lia*o2*345/*o2*-1 and lia*o2*345/*o2*-2 were developed from a cross between lia*o2*345 X CA339 by using SSR marker *phi057* and sequence and transcript abundance analyses indicated that *o2* transcript was largely inhibited and they concluded that different crossing over patterns during the process of *o2* NILs construction resulted in the different kernel phenotypes of the two *o2* NILs which showed that *o2* mutant gene possesses differential expression due to insertion of transposable element (Chen *et al*., 2014).

Mapping analysis for identification of QTLs in *o2* which influencing the tryptophan content and candidate gene based SSRs for their utilization in MAS was done with population of 218 $F_{2:3}$ individuals from a cross between VQL2 (low tryptophan content) and VQL8 (high tryptophan content). The lowest tryptophan content based on % of protein was 0.162, while the highest value was 0.905 with a mean of 0.353. Single Marker Regression (SMR), Simple Interval Mapping (SIM) and CIM analysis performed for $F_{2:3}$ populations and they identified five significant QTLs on

chromosomes 5, 7 and 9. Also they found that *Wx1* gene which influences the amino acid composition of the maize endosperm was mapped on chromosome 9 near the marker *phi022* and lys-sensitive Asp kinase was found to be a better candidate gene for QTL which affects free amino acid content in maize (Babu *et al*., 2015).

Tufchi *et al.* (2015) studied the effect of *o2* allele on accumulation of tryptophan among backcross-derived introgressed progenies of maize to develop new inbreds for further use in breeding programme and development of QPM hybrid. $BC_2F_{2:3}$ population from a cross between Pant 10k 1375 x CML161 lines were used for determination of tryptophan concentration where variable expression of *o2* gene as seen from the variability in tryptophan concentration (0.046-.082 %). They reported that considerable variation between normal maize and *o2* lines in tryptophan amount but none of the progenies had tryptophan concentration higher than the donor line CML161. They suggested that progenies with tryptophan concentration greater than 0.075 % could serve as potential germplasm in development of QPM hybrids.

Development of high tryptophan maize NILs adapted to temperate regions through MAS was carried out by Kostadinovic *et al.* (2016). In this study CML 144 as donor parent to convert ZPL 3 and ZPL 5 into QPM versions in which most plants recovered with 93 % of recurrent parent genome and increase of 30 % in tryptophan content, 36 % in QI as well as kernels with less than 25 % opaque endosperm lines, improved grain yield and inbred lines with good combining abilities were identified. These lines could be adapted to the temperate climate to mitigate adaption problems which increased the efficiency of MAS in different breeding programmes.

Conversion of elite maize inbred line BML-7 into QPM with high lysine and tryptophan using MAS was carried out by Krishna *et al.* (2017) by using QPM line CML-186 (donor parent) and identification of polymorphism between donor and recipient parent was done by *o2* gene specific SSR markers *viz. umc1066*, *phi057* and *phi112.* Among 200 plants in BC_2F_1, three plants were found to be recovered with 90-93 % recurrent parent genome by two generations of back crossing for *o2* and amino acid modifiers in the BML-7 genome and those lines were forwarded for agronomical and biochemical evaluation to confirm and identify the QPM line which led to development of QPM version of BML-7 with 0.97 % of tryptophan and 4.04 % of lysine concentration in protein.

Sangamitra *et al.* (2017) studied genetic purity of four QPM hybrids and their parental lines by analyses with biochemical markers *viz.* alcohol dehydrogenase

(ADH), esterase (EST), acid phosphatase (ACP) and malate dehydrogenase (MDH) and 30 selected SSR markers. Isoenzymes are being co-dominant in nature could be able to detect heterozygous in F1 hybrid by which parental lines were distinguished from hybrids but off types present within the parents could not able to detect. So based on results of analyses with biochemical markers, lines were forwarded for molecular characterization with 30 SSR markers. In molecular studies only dupssr34 could able to distinguish the parental lines from hybrids by polymorphism and showed 87.5 % purity in hybrid which could suggest that efficient utilization of dupssr34 in maize breeding programme.

Development of commercial QPM inbred lines which could be productive in various environmental conditions was carried out by study on a set of forty maize inbred lines (QPM and non-QPM). These lines were examined for molecular, biochemical and morphometric analysis where genotypes were grouped (Mahalanobis distance-based clustering) on the basis of flowering and yield traits and the genetic relationships among the genotypes were also analysed by Principal Coordinate Analysis (PCA). Among the non-QPM set, two early maturing lines BAJIM-08-26 and KI-30 were found superior for grain yield and among QPM set, CML189 line was found to be superior for high tryptophan content and these lines identified as well-adapted agronomically superior lines and suggested to be a better donor in QPM development breeding programmes (Sood *et al*., 2017).

Singh *et al*. (2017) worked on introgression of *o2* allele from QPM inbred lines to normal inbred lines in which four QPM inbred lines and eight normal maize inbred lines were taken for introgression and *o2* introgressed lines were confirmed by the *o2* SSR markers *phi057*, *umc1066* and *phi112.* Among various cross combinations between normal maize and QPM, they identified two cross combinations V335 × CML 141 and V351 × CML 141 and forwarded for tryptophan and lysine estimation which showed that 81 to 102 % increase in protein quality with desirable agronomic traits.

Surender *et al*. (2017) studied about development of QPM version of DHM117 maize hybrid (BML6 x BML7) by using CML 181 as donor parent. In this study *o2* gene based SSR markers *viz. umc1066, phi057* and *phi112* were used to check the polymorphism between donor and recipient parents where *umc1066* and *bnlg1200, bnlg2160* used for foreground selection and recombinant selection respectively. Converted lines of BML6 and BML7 lines with higher tryptophan content notified as CB6 and CB7 respectively and among all cross combinations of these lines with donor parents only CB6-36 × CB7-28 and CB6-36 × CB7-59 exhibited relatively higher tryptophan content with acceptable grain yield per plant improvement compared to

check DHM 117 which needs to be tested further for multi-location trials to improve adaptability.

Use of *o2* gene specific markers provided an opportunities to identify the converted elite inbreds with homozygous *o2o2* genes through MAS whereas in this study two elite normal maize inbreds OML 17-3 and OML 42-9 with 45 % yield advantage compared to standard check Vivek QPM 9, used for conversion to QPM using *o2* donors CML 176 and CML 186. Results of molecular divergence studies with *phi057* on BC_3F_1 population concluded that OQL 176-17-3 and OQL 186-42-9 lines developed using marker assisted back cross breeding with good agronomical characters on par with QPM donors (CML 176 and CML 186) and nearly double the lysine and tryptophan content as compared to respective normal inbreds (OML 17-3 and OML 42-9) (Tripathy *et al*., 2017).

Tandzi *et al.* (2017) reviewed importance of breeding of QPM suitable for stress conditions and constraints in adoption of QPM by farmers. Evaluation of QPM in various locations has proved that stability of lysine and tryptophan content. Even though emphasis was placed on screening of QPM for heat, cold, soil pH, salinity and also pest and diseases, but still more emphasis should be given to breeding QPM for tolerance to biotic and abiotic stress. Also adoption of QPM genotypes by farmers has been found to be limited mainly due to the minimal dialogue between maize breeders and farmers. So participatory approaches in the breeding process could be effective way to draw their attention to available nutritional benefits in QPM and also QPM growers need to be trained and informed about the challenges facing in QPM production as well as to overcome these challenges.

Pandey *et al*. (2018) studied about diversity among the haplotypes present in the forty six QPM inbreds from diverse region of India and CIMMYT which varied for endosperm modification and amino acid concentration by *o2* gene specific SSR markers *umc1066* and *phi057*. Total of 5 alleles were observed which included 2 alleles from *umc1066* and 3 alleles from *phi057* and various frequency of haplotype combination observed for these 2 markers. Among various combination of haplotypes, *o2*-BD was found to be the most promising haplotype combination for improved tryptophan and lysine content which obtained only 4.35 % of total population in this study and also considerable variation between CIMMYT lines and other commercial lines developed from India showed by different haplotype combination and these combinations could be used to identify the elite germplasms for effective breeding programme.

Sarika *et al*. (2018) studied about action of novel *opaque16* (*o16*) genes in four elite inbred lines and four commercial QPM maize hybrids adapted to subtropical and diverse agro-ecological zones of India. In this study *o16* introgressed into *o2* carrying inbred lines and commercial QPM hybrids by Marker Assisted Backcross Breeding (MABB) with *phi057, phi112* and *umc1066* markers for *o2* and *umc1141* and *umc1149* markers for *o16*. Selected *o16* introgressed lines based on the maximum recovery of recurrent parent genome showed phenotypic resemblance with their recurrent parents in plant and kernel characteristics and some of pyramided lines with *o2* and *o16* showed increased lysine and tryptophan content than their recurrent parents but moderate variation in multi-location trials which revealed that *o16* introgression could be as a supplementary for nutritional improvement in maize and effective tool in QPM development breeding programme.

Biochemical analysis of QPM inbred lines followed by molecular validation was done by Tripathy *et al*. (2018) with ten Odisha QPM Lines (OQL) with improved lysine and tryptophan content, ten normal maize lines and two standard QPM lines from CIMMYT. Seed protein content and essential amino acids tryptophan and lysine content was estimated for all these twenty lines which showed that two OQL inbreds OQL 17-3 and OQL 42-9 with increased seed crude protein and also enriched with tryptophan and lysine content. Selected lines forwarded for molecular validation with *o2* specific SSR markers *phi057, phi112* and *umc1066* and selected OQL confirmed as QPM by co-dominant marker *phi057* with improved quality characters.

Chapter 3
Materials and Methods

The experimental work was carried out at College of Post Graduate Studies, Umiam, Meghalaya. Sib matings on standing $F_{2:3}$ families were done in May 2017 and $F_{3:4}$ generation was grown during July 2017. Sib matings within these $F_{3:4}$ families and biochemical and molecular characterization of harvested cobs was then followed up.

3.1 Plant material

The $F_{3:4}$ generation was developed by successive sib matings from F_2 onward of half diallel matings previously developed using QPM inbred lines CML 161, CML 165, V341, V373 and V390 obtained from CIMMYT, India. The half diallel matings and evaluation had been carried out by Backiyalakshmi (2016) while the families derived were advanced to $F_{2:3}$ by Khati (2018) as part of their M.Sc. research programme. For the current study, from a total four hundred and seventy three sib matings obtained from $F_{2:3}$ families, sixty QPM families selected on the basis of kernel vitreousness and higher seed index value were used to raise the $F_{3:4}$ generation and further studies were done.

3.2 Methodology

3.2.1 Layout and design of the experiment

The selected families were planted in ear to row fashion replicated twice in a Randomized Block Design (RBD) with each row comprising of ten plants in July 2017. Plant to plant spacing was kept at 20 cm and row to row spacing was maintained at 60 cm. Routine package of practices and intercultivation operations were carried out.

3.2.2 Observations Recorded

Yield contributing traits as well as other morphological and qualitative traits were recorded for the $F_{3:4}$ generation. Each sib mated cob was harvested separately. The different traits recorded were

Days to 50 % tasseling: It was recorded as the number of days from germination to when 50 % of the plants shed pollen.

Days to 50 % silking: It was recorded as the number of days from germination to when 50 % of the plants showed up 2 to 3 cm long silk protrusion.

Anthesis-Silking interval: It was calculated from the difference between number of days to 50 % silking and days to 50 % tasseling.

Plant height (cm): It was measured from ground level of plant stalk to base of leaf sheath of the matured plant (post flowering) and it was expressed in centimetre.

Ear height (cm): It was measured from ground level of plant stalk to the node bearing upper most ear and it was expressed in centimetre.

Weight of the cobs with husk (g): It was recorded by weighing the cobs with husk and expressed in grams.

Weight of the cobs without husk (g): It was recorded by weighing the dehusked cob and expressed in grams.

Cob length (cm): The length of the cob was measured from base to tip and expressed in centimetre.

Cob diameter (cm): Width of the cob measured and expressed in centimetre.

Number of kernels rows per cob: Data on number of kernel rows per cob counted and expressed as an integer.

Number of kernels per row: Data on number of kernels in each row counted and the average expressed as an integer.

Seed Index (g): This was calculated by weighing 100 filled grains and expressed in grams.

Grain yield per plant (g): This was calculated by weighing the total number of grains of individual cob of particular plant and it was expressed in grams.

Several qualitative characters were also studied based on the presence or absence of the trait. Similarly disease incidence was also scored.

The traits studied were as follows:

Tassel anthocyanin– presence/absence: Presence of tassel anthocyanin indicates purple colour on tassel branches. Based on presence or absence of anthocyanin scores were given and it is expressed as an integer (Table 3.1).

Table 3.1 Scores for presence or absence of tassel anthocyanin

Type	Score
Presence	1
Absence	0

Silk anthocyanin– presence/absence: Presence of silk anthocyanin indicates purple colour on silk protruding from cob. Based on presence or absence of anthocyanin scores were given and it is expressed as an integer (Table 3.2).

Table 3.2 Scores for presence or absence of silk anthocyanin

Type	Score
Presence	1
Absence	0

Attitude of lateral branches: It was graded by the angle between main spikelet and their lateral branches. For different altitude, scores were given in Table 3.3.

Table 3.3 Types of altitudes of lateral branches

Type	Score
Straight	1
Curved	2
Fully curved	3

Density of spikelet: Number of spikelets was counted and scores were given for each type of densities. It is expressed in integer (Table 3.4).

Table 3.4 Spikelet density type

No. of spikelets	Type	Score
0-5	Less	1
6-10	Medium	2
>10	Dense	3

Leaf angle: Leaf angle was categorized based on the angle between leaf stalk and plant stalk. It is expressed as an integer (Table 3.5).

Table 3.5 Various leaf angles

Type	Score
Straight	1
Curved	2
Fully curved	3

Diseases-presence/absence: It was categorized based on the presence of disease symptoms and expressed as an integer (Table 3.6).

Table 3.6 Disease scoring table

Type	Score
Presence	1
Absence	0

Stalk anthocyanin - presence/absence: Presence of stalk anthocyanin indicates purple colour on stem. Based on presence or absence of anthocyanin scores were given and it is expressed as an integer (Table 3.7).

Table 3.7 Scores for presence or absence of stalk anthocyanin

Type	Score
Presence	1
Absence	0

Stalk hair– presence/absence: It was categorized depends on the presence of stalk hair. Scores were given for this parameter and it is expressed as an integer (Table 3.8).

Table 3.8 Scores for presence or absence of stalk hair

Type	Score
Presence	1
Absence	0

Kernel colour: Kernel colour categorized as white, yellow and brown. For each colour, scores were given and expressed as an integer (Table 3.9).

Table 3.9 Kernel colour classification

Colour	Score
White	0
Yellow	1
Brown	2

Kernel texture: Kernel texture accordingly rated as flint, semi-flint and dent based on appearance. For kernel textures, scores were given and the graded textures were expressed as an integer (Table 3.10).

Table 3.10 Kernel texture classification

Texture type	Score
Flint	1
Semi-flint	2
Dent	3

Kernel row arrangement: Cobs were graded as straight, spiral and irregular based on the kernel row arrangement and expressed as an integer (Table 3.11).

Table 3.11 Kernel row classification

Row arrangement type	Score
Straight	1
Spiral	2
Irregular	3

3.2.3 Screening for hard endosperm using light box table

Six hundred and eighty one cobs obtained from harvesting $F_{3:4}$ families individually were subjected to light table screening for kernel vitreousness by visually grading the endosperm based on opacity as per Vivek *et al.* (2008). Gradation in opaqueness was scored on a 1 to 5 scale (Table 3.12). Type 1 kernels are completely translucent, with no opaqueness and *o2o2*-induced softness is simply not present. Type 5 kernels with very soft endosperm, complete opaqueness and no modifiers, results in undesirable characters such as susceptibility to ear rots and weevils and kernel cracking. Less opaqueness implies higher/more action of modifiers. Therefore selection was done for lines with scores of type 2 and 3.

Table 3.12 Modification score for opaqueness

Sl. No.	Scores	Opaqueness
1.	Type (Modification score) 1	0 % opaque
2.	Type (Modification score) 2	25 % opaque
3.	Type (Modification score) 3	50 % opaque
4.	Type (Modification score) 4	75 % opaque
5.	Type (Modification score) 5	100 % opaque

Based on light box screening, sixty $F_{3:4}$ lines with scores of 2 and 3 and higher seed index were further advanced for tryptophan estimation.

3.2.4 Tryptophan estimation

Colorimetric assay for tryptophan content was done based on Hopkins-Cole reaction (Vivek *et al*., 2008), using the following reagents.

Reagent A (Ferric chloride six hydrated glacial acetic acid): 270 mg of $FeCl_3.6H_2O$ dissolved in 1 litre of glacial acetic acid.

Reagent B (30 N sulfuric acid): 833.3 ml of sulfuric acid (98 %) and 166.7 ml of distilled water to mixed at the same time to prepare 30 N H_2SO_4 solutions.

Reagent C (Reagent A+ Reagent B): Prepared by volume to volume mixture of reagents A and B at least one hour prior to use.

Papain solution (4 mg/ml): 40 mg of papain weighed and dissolved in 10 ml of sodium acetate solution at room temperature to 10 ml of papain solution.

The principle used in analysis was based on one molecule of glyoxylic acid and two molecules of tryptophan reacting to form a coloured compound with a maximum absorption at 560 nm. From the selected sixty lines, 20-25 seeds were ground to a very fine powder and the sieved flour of each genotype was wrapped in a piece of commercial filter paper followed by defattening with hexane in a Soxhlet-type continuous extractor for 6 hours. After hexane evaporation from the defatted maize flour, 80 mg of powder was digested using 3 ml of 4 mg/ml papain. The samples were then incubated for 16 hours at 65^0 C and centrifuged at 6500 rpm for 10 min followed by another round of centrifugation at 2500 rpm for 5 min. 1 ml of the hydrolysate was then transferred into a new tube and mixed with 4 ml of reagent C and incubated for 15 min.

The optical density (OD) of samples was recorded at 560 nm in a UV-Visual Double Beam Spectrophotometer. Each sample was analyzed in triplicate to ensure accuracy. A stock solution of 100 µg/ml of DL-tryptophan was in 0.165 M sodium acetate at pH 7 and stored at 4^0C. The solution was diluted with sodium acetate to 0, 10, 15, 20, 25, and 30 µg of tryptophan per ml to calibrate and plot a standard curve (Fig. 3.1) with the absorbance readings as a function of concentration for calculating the slope (y) of the standard curve.

Calculations of tryptophan percentage were done as follows:

$$\% \text{ trp} = \frac{OD_{560\text{ nm}}}{\text{slope}} X \frac{\text{hydrolysis volume}}{\text{sample weight}} X\ 100\%$$

$$\% \text{ trp} = OD_{560\text{ nm corrected}} \text{ x Factor}$$

Where:

$$OD_{560\text{ nm corrected}} = OD_{560\text{ nm sample}} - OD_{560\text{ nm average of papain blanks.}}$$

$$Factor = \frac{0.00375}{\text{slope}}$$

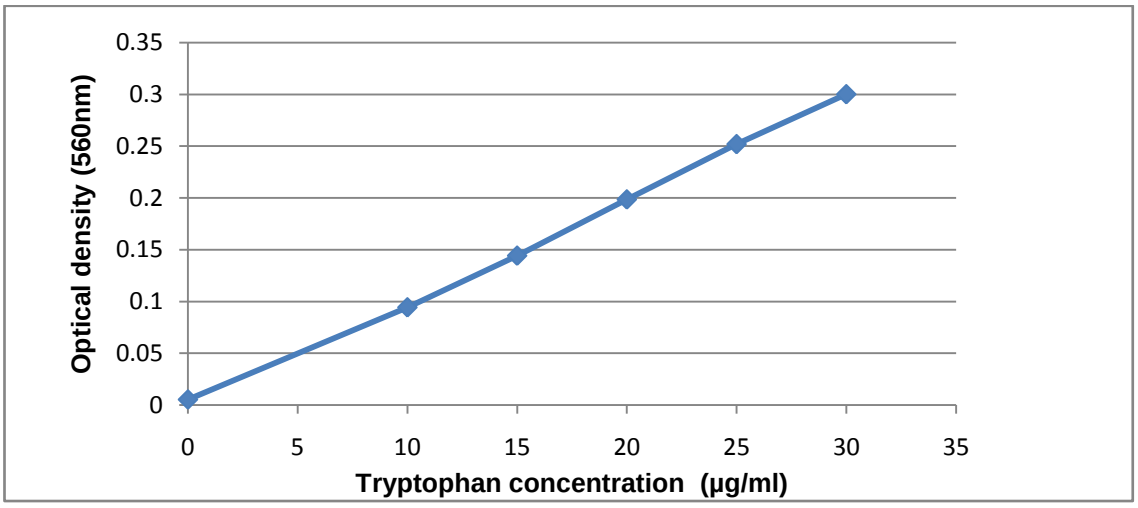


Fig. 3.1 The calibrated standard curve using known values of tryptophan concentration

3.2.5 Molecular characterization of selected $F_{3:4}$ QPM lines

Characterization of selected QPM lines at the DNA level was done using reported SSR markers for *o2 specific and associated* and *o2* modifiers. Leaf samples were collected from 15 day old for genomic DNA extraction.

3.2.5.1 Extraction of DNA:

DNA isolation from young and healthy leaves of 15 day old seedlings stored at -20°C was done using the CTAB method (Doyle and Doyle, 1990) with slight modifications. 0.5 g leaf sample was homogenised in 1 ml of freshly prepared 2 % CTAB extraction buffer [25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 2 %

CTAB, plus 0.4 % β-mercaptoethanol]. The homogenate was then transferred to 1.5 ml microcentrifuge tubes and incubated at 65ºC for 45 min and gently mixed by inverting the tubes at an interval of 5 minutes. Thereafter the tubes were centrifuged at 5000 rpm for 5 minutes. 800 µl of supernatant was collected and equal amount of chloroform- isoamylalcohol (24:1) was added to the tubes and gently mixed for 1 minute. The samples were then centrifuged for 15 minutes at 13,000 rpm and 800 µL of the supernatant transferred to a fresh tube to which 2/3 volume of cold isopropanol (-20ºC) had been added. The samples were gently mixed and again centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and the DNA pellet washed with 500 µl of 70 % ethanol to eliminate salt residues adhered to the DNA, and the sample was dried for approximately 1 hour at room temperature. The pellet was then resuspended in 40 µl 1X TE (10 mM Tris**-**HCl, pH 8.0 and 1 mM EDTA, pH 8.0) and stored at -20ºC.

3.2.5.2 Determination of quality of DNA:

The quality of the DNA was determined by loading 3 µl of DNA with 2 µl of loading dye along with 1 µl of 1 kb ladder (GeneRuler, Fermentas) in a horizontal 0.8 % agarose gel. Depending on quality of the DNA obtained, the working solution was made by diluting genomic DNA using 1X TE buffer. Dilution of the genomic DNA for the present study was done at a concentration of 1:5 (10 µl of DNA in 40 µl of TE buffer) and 1:5 (10 µl of DNA in 90 µl of TE buffer). The composition of stock TE and TBE buffers used in the present study are shown in Table 3.13 and Table 3.14, respectively.

Table 3.13 Components of 100 ml 10X TE (Tris-EDTA) buffer

Sl. No.	Components	Volume (ml)	Final Concentration
1	1 M Tris HCl (pH 8)	10	100 mM
2	0.5 M EDTA (pH 8)	2	10 mM
3	Distilled water	88	

Table 3.14 Components of 1 L 10X TBE stock solution

Sl. No.	Components	Amount
1	Tris Base	108 g
2	Boric acid	55 g
3	0.5 M EDTA (pH 8)	40 ml
4	Distilled water	797 ml

3.2.5.3 SSR markers for *o2* and *o2* modifiers:

A set of 20 SSR markers for *o2* and *o2* modifiers were used for molecular studies of the selected QPM lines in the present study (Table 3.15).

Table 3.15 List of SSR markers used in this study

Sl. No	SSR marker	Forward primer	Reverse primer
1.	*umc1695*	CAGGTAATAACGACGCAGCAGAA	CAGGTAATAACGACGCAGCAGAA
2.	*bnlg2132*	GGCGAGAGAGGCAAAGTTAA	GTCGCACAAGGGGATCAC
3.	*phi057*	CTCATCAGTGCCGTCGTCCAT	CAGTCGCAAGAAACCGTTGCC
4.	*phi112*	TGCCCTGCAGGTTCACATTGAGT	AGGAGTACGCTTGGATGCTCTTC
5.	*umc1066*	ATGGAGCACGTCATCTCAATGG	AGCAGCAGCAACGTCTATGACACT
6.	*umc1929*	TCCAAGCATTATGCATCACAAACT	GCATGAATTCAACACAGAACCACT
7.	*umc1393*	CCTTCTTCTTATTGTCACCGAACG	GCCGATGAGATCTTTAACAACCTG
8.	*umc1036*	CTGCTGCTCAAGGAGATGGAGA	GACACACATGCACGAGCAGACT
9.	*umc1978*	CACTCCCTCCATTTCTCTCACCT	ATCGCTACCATTGGACGCTTTAC
10.	*bnlg1200*	CGTCCTCGTTGTTATTCCGT	GTTCCCTCTCTCCCTCCCTC
11.	*bnlg2160*	GAAGCAACCCATTTTCATCC	AGATTGGATTCCTGCCTCCT
12.	*phi034*	TAGCGACAGGATGGCCTCTTCT	GGGGAGCACGCCTTCGTTCT
13.	*umc1831*	TTTCGACTGCTAGTGTACTTGGGG	CTCTACATCTTCAGCGTCTCCACA
14.	*umc1016*	GTGATACCGGGTAATCTGGTGC	GATGATGGGTGATCATCGGTTC
15.	*bnlg1164*	AAACAGGGTGTGACAGGTCC	GAACGGGCAGACGCATAAG
16.	*bnlg1179*	GCGATTCAGTCCGCAGTAGT	GTACTGAACAAACCGTGGGC
17.	*bnlg1643*	ATTGACCCCGTGACCCTC	ACCACCGTCCACCTCCAC
18.	*bnlg1633*	TCAACTTCTCATGCACCCAT	GTACCTCCAGGTTTACGCCA
19.	*bmc2136*	TGCTCCTTCTCGAGCACC	ATGGACGTACGGCAGACTCT
20.	*umc1357*	TAGACATGTTGAAACCAGGACCG	ACGACGTCAACAACAGCATGA

3.2.5.4 Polymerase Chain Reaction (PCR):

PCR was carried out with SSR markers in a reaction volume of 10 µl. The components of PCR master mix, volumes and their concentration used are indicated in Table 3.16.

The PCR master mix for 10 µl reaction consisted of 2 µl of genomic DNA, 0.6 µl each of forward and reverse primer, 1 µl of deoxynucleoside triphosphate (dNTP), 0.08 µl of Taq polymerase, 0.6 µl of $MgCl_2$, 1 µl of 10x PCR buffer. 4.7 µl of PCR water was added and volume made up to 10 µl by adding PCR water. The amplification was carried out in Thermo cycler using different PCR profiles. After PCR reaction, the samples were kept at hold for 10 minutes and after final extension the PCR products were stored in -10^0C.

The optimal cycling parameters for amplification were initial denaturation at 94^0C for 5 minutes, followed by 94^0C for 30 seconds of final denaturation, 30 seconds at either 52, 55, 56, 57, 58, 59, 60^0C (depending on the SSR marker) of annealing, 45 seconds at 72^0C of extension followed by a 10 min final extension at 72^0C. The entire steps were performed for 35 cycles. The amplified product was stored at 4^0C.

Table 3.16 Composition of PCR Master mix

Sl. No.	PCR component	Volume (µl)	Final Concentration
1.	Genomic DNA	2	100-150 ng
2.	dNTPs (2mM)	1	0.2 mM
3.	Forward primer (10µM)	0.6	0.6 µM
4.	Reverse primer (10µM)	0.6	0.6 µM
5.	$MgCl_2$ (25mM)	0.6	1.5 mM
6.	DNA Taq polymerase (Sigma) (5 Units/µl)	0.08	0.040 Units/ µl
7.	PCR buffer (10X)	1 (5X)	0.5X
8.	PCR water	4.7	-
	Reaction volume (Total)	10 µl	

3.2.5.5 Resolution of amplified PCR products:

The PCR products were analyzed by electrophoresis using a 2 % agarose gel to which was added 5 µl of ethidium bromide (10mg/ml) in a running tank containing 0.5 X TBE buffer. The PCR amplified products were mixed with 3X loading dye and loaded into the wells. The samples were run at 90 V for 2 to 2 ½ hours. A 100 bp ladder was added to help determine the size of amplified fragments. The gel was visualized under UV-transilluminator and documented using ALPHA IMAGER gel documentation system (M/s Alpha innotech) which was stored for further scoring.

3.2.5.6 Scoring of PCR products:

The size of the amplified products was determined and scoring was done manually based on the size of the amplified products. The alleles were assigned band sizes relative to the ladder and scored using letters A, B, C, D, E and F which represent different alleles while zero (0) represents absence of the allele. Based on this data Polymorphic Information Content (PIC) of each marker was calculated as described by Smith *et al*. (1997).

3.2.5.7 Cluster analysis:

By using the marker data for all maize genotypes under study, dendrogram was constructed with Unweighted Pair Group Method with Arithmetic mean (UPGMA) using Darwin 6.0.15 and dissimilarity matrix between the genotpyes based on molecular data was computed using Jaccard's coefficient (1908) and this matrix was analyzed using NTSYS-pc 1.70 to produce an agglomerative hierarchial classification by UPGMA.

3.2.6 Statistical analysis

The following statistical tools were used for interpreting the data generated in all the experiments:

3.2.6.1 Mean:

The mean value for the different traits was calculated as:

$$\overline{X} = \frac{\sum X}{N}$$

Where, X is the sample mean ΣX = Sum of all the observations, N= total number of samples studied.

3.2.6.2 Range and Standard Deviation:

The minimum and maximum values and the standard deviation were calculated.

Standard Deviation was calculated as:

$$s = \sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}}$$

Where, s= standard deviation, X is the sample mean n= total number of samples studied.

3.2.6.3 Standard Error:

The Standard Error for the different traits studied with respect to mean was also calculated as:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Where, s=standard deviation, N= total number of samples studied.

3.2.6.4 Selection differential (S):

The Selection differential for tryptophan was calculated as:

$$\mathbf{K = Xm - Xb}$$

Where,

K = Selection Differential

Xm = Mean tryptophan value of particular family

Xb = Base population mean

3.2.6.5 Genetic gain (R):

The genetic gain for tryptophan was calculated as:

$$\mathbf{R = Xi - Xb}$$

Where,

R = Genetic gain

Xi = Individual tryptophan value

Xb = Base population mean

3.2.6.6 Realized Heritability:

Realized heritability for tryptophan was calculated as:

$$\boldsymbol{Realized\ Heritability} = \frac{\boldsymbol{Genetic\ Gain\ (R)}}{\boldsymbol{Selection\ Differential\ (S)}}$$

3.2.6.7 Polymorphic Information Content (PIC):

Polymorphic Information Content for different markers was calculated as:

$$\boldsymbol{Polymorphic\ Information\ Content} = \mathbf{1} - \sum \boldsymbol{pi^2}$$

$$\boldsymbol{pi} = \frac{\boldsymbol{No\ of\ bands\ from\ the\ particular\ allele}}{\boldsymbol{Total\ no.of\ bands}}$$

3.2.6.8 Heterozygosity:

Heterozygosity for different markers was calculated as:

$$\mathbf{Heterozygosity}(\%) = \frac{\mathbf{No\ of\ heterozygous\ bands}}{\mathbf{Total\ no.of\ bands}} * \mathbf{100}$$

3.2.6.9 Genotypic variance (Vg):

Genotypic variance for different traits was calculated as:

$$\mathbf{Genotypic\ variance} = \frac{\mathbf{Treatment\ MSS - Error\ MSS}}{\mathbf{Replication}}$$

Where,

Treatment Mss = Treatment mean sum of squares

Error Mss = Error mean sum of squares

3.2.6.10 Phenotypic variance (Vp):

Pheontypic variance for different traits was calculated as:

$$\mathbf{Phenotypic\ variance} = \mathbf{Genotypic\ variance} + \mathbf{Environmental\ variance}$$

$$\boldsymbol{V_P} = \boldsymbol{V_G} + \boldsymbol{V_E}$$

3.2.6.11 Genotypic Covariance (GCV):

Genotypic Covariance for different traits was calculated as:

$$\mathbf{GCV} = \frac{\sqrt{\mathbf{Genotypic\ variance}}}{\mathbf{mean}} \times \mathbf{100}$$

3.2.6.12 Phenotypic Covariance (PCV):

Phenotypic Covariance for different traits was calculated as:

$$\mathbf{PCV} = \frac{\sqrt{\mathbf{Phenotypic\ variance}}}{\mathbf{mean}} \times \mathbf{100}$$

3.2.6.13 Heritability:

Heritability for different traits was calculated as:

$$\mathbf{Heritability} = \frac{\mathbf{Genotypic\ variance}}{\mathbf{Phenotypic\ variance}} = \frac{\mathbf{V_g}}{\mathbf{V_p}}$$

3.2.6.14 Cob diameter (cm):

Cob diameter for individual cobs was calculated as:

$$\mathbf{Cob\ diameter\ (cm)} = \frac{\mathbf{Cob\ girth}}{\mathbf{3.1416}}$$

Chapter 4

Results

Six hundred and eighty one sib mated lines of $F_{3:4}$ generation were studied for selecting QPM lines with higher tryptophan content based on kernel vitreousness, high tryptophan percent followed by molecular validation using SSR markers at the DNA level.

The results obtained are presented under the following heads:

4.1 Screening for kernel vitreousness along with high seed index.

4.2 Tryptophan content estimation of selected $F_{3:4}$ families using Hopkins-Cole reaction.

4.3 Genetic Gain (R), Selection Differential (S) and Realized Heritability (R/S) in $F_{3:4}$ families for tryptophan content.

4.4 Characterization of $F_{3:4}$ yield, yield contributing traits and quality traits.

4.5 Molecular characterization of selected $F_{3:4}$ QPM sib families

4.1 Screening for kernel modification with higher seed index

For the $F_{3:4}$ families generated from selfing F_2 population of the cross V 341 X V 373, thirty four sib matings were successfully effected. A total of one hundred seven sib matings could be effected for families derived from the cross CML 165 X V 373. Another one hundred twenty four and four hundred and sixteen sib matings were successful for $F_{3:4}$ families derived from the crosses CML 161 X V 341 and CML 161 X V 373 respectively. In all, six hundred and eighty one sib mated harvested cobs were screened for kernel modification as per Vivek *et al*. (2008). Four hundred and forty one sib mated lines recorded an acceptable kernel score of 2/3 (Table 4.1). Of these 60 lines with the highest seed index value (Fig. 4.1) (Table 4.2) were selected for percent tryptophan estimation.

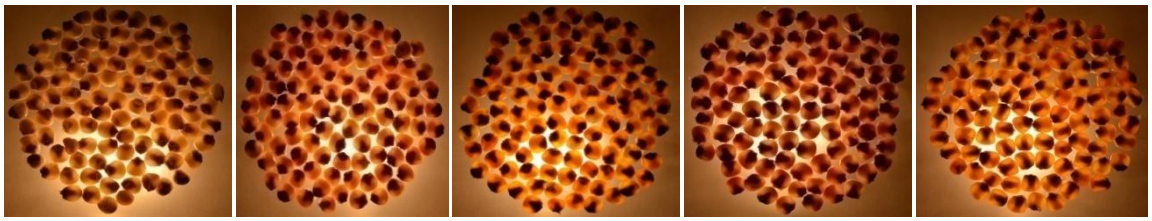
Table 4.1 Grouping of $F_{3:4}$ sib mated lines based on kernel modification score

Sl. No	Kernel Score	Number of sib mated lines
1.	Score 2	149
2.	Score 3	292
3.	Score 4	204
4.	Score 5	36
		TOTAL: 681

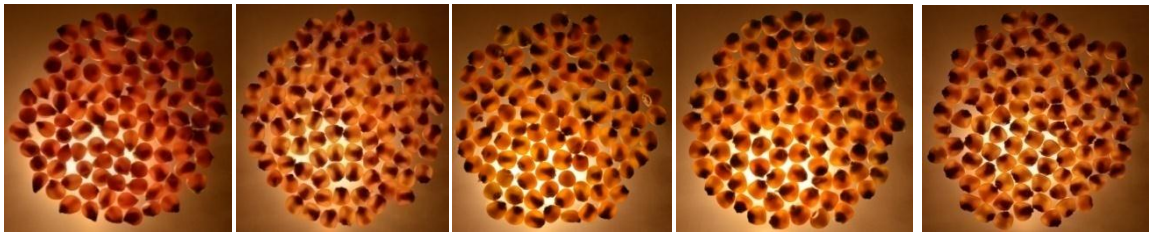
Table 4.2 Light box score and seed index for the selected sixty lines

Sl. No.	Genotype Code	Kernel Score	Seed Index (g)	Sl. No.	Genotype Code	Kernel Score	Seed Index (g)
1	UMQ 161x341-1	2	40.7	31	UMQ 161x373-10	3	35.5
2	UMQ 161x341-2	2	39.2	32	UMQ 161x373-11	3	33.4
3	UMQ 161x341-3	3	37.6	33	UMQ 161x373-12	2	33
4	UMQ 161x341-4	3	37	34	UMQ 161x373-13	3	32.1
5	UMQ 161x341-5	3	36	35	UMQ 161x373-14	3	32
6	UMQ 161x341-6	3	34.4	36	UMQ 161x373-15	3	31.8
7	UMQ 161x341-7	3	33.7	37	UMQ 161x373-16	3	31.5
8	UMQ 161x341-8	3	33.5	38	UMQ 161x373-17	3	30.2
9	UMQ 161x341-9	3	33.2	39	UMQ 161x373-18	3	30
10	UMQ 161x341-10	3	32.5	40	UMQ 161x373-19	3	29.8
11	UMQ 161x341-11	2	41.5	41	UMQ 161x373-20	3	29.5
12	UMQ 161x341-12	3	31.5	42	UMQ 161x373-21	3	29.4
13	UMQ 161x341-13	3	30.8	43	UMQ 161x373-22	3	29.3
14	UMQ 161x341-14	3	30.5	44	UMQ 161x373-23	3	29
15	UMQ 161x341-15	2	30.3	45	UMQ 161x373-24	3	29
16	UMQ 161x341-16	3	30.2	46	UMQ 161x373-25	2	28.8
17	UMQ 161x341-17	3	29.7	47	UMQ 161x373-26	3	28.8
18	UMQ 161x341-18	3	29.2	48	UMQ 161x373-27	2	28.8
19	UMQ 161x341-19	2	28.5	49	UMQ 161x373-28	3	28.4
20	UMQ 161x341-20	2	28.2	50	UMQ 161x373-29	3	28.3
21	UMQ 161x341-21	2	28.1	51	UMQ 165x373-1	3	33.8
22	UMQ 161x373-1	3	32.2	52	UMQ 165x373-2	3	33.8
23	UMQ 161x373-2	3	40.6	53	UMQ 165x373-3	3	32.7
24	UMQ 161x373-3	3	40.3	54	UMQ 165x373-4	3	30.8
25	UMQ 161x373-4	3	40.1	55	UMQ 165x373-5	2	30.2
26	UMQ 161x373-5	2	40	56	UMQ 165x373-6	3	30
27	UMQ 161x373-6	3	38.3	57	UMQ 165x373-7	3	29.5
28	UMQ 161x373-7	3	37.8	58	UMQ 165x373-8	3	20.4
29	UMQ 161x373-8	3	37.2	59	UMQ 165x373-9	2	29
30	UMQ 161x373-9	3	35.7	60	UMQ 341x373-1	3	27.7

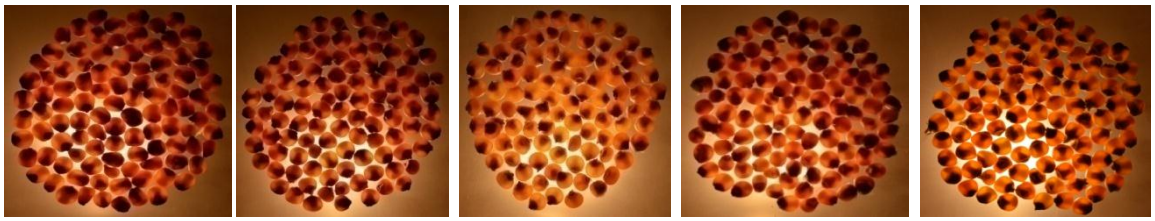
Fig. 4.1 Light box images and respective scores for the selected sixty lines in the $F_{3:4}$ generation



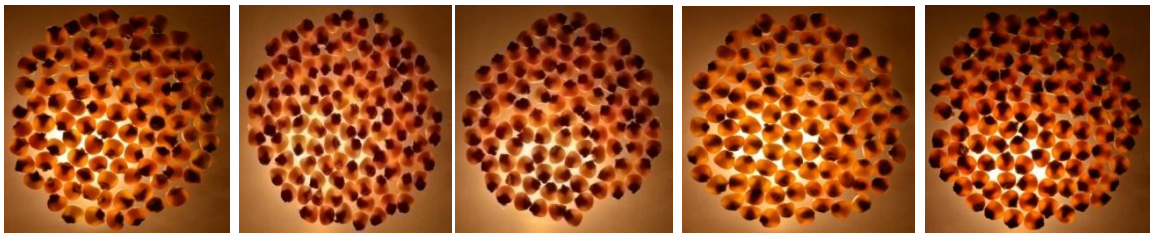
UMQ 161x341-21 Kernel score: 2 | UMQ 161x373-1 Kernel score: 3 | UMQ 161x373-2 Kernel score: 3 | UMQ 161x373-3 Kernel score: 3 | UMQ 161x373-4 Kernel score: 3



UMQ 161x373-5 Kernel score: 2 | UMQ 161x373-6 Kernel score: 3 | UMQ 161x373-7 Kernel score: 3 | UMQ 161x373-8 Kernel score: 3 | UMQ 161x373-9 Kernel score: 3

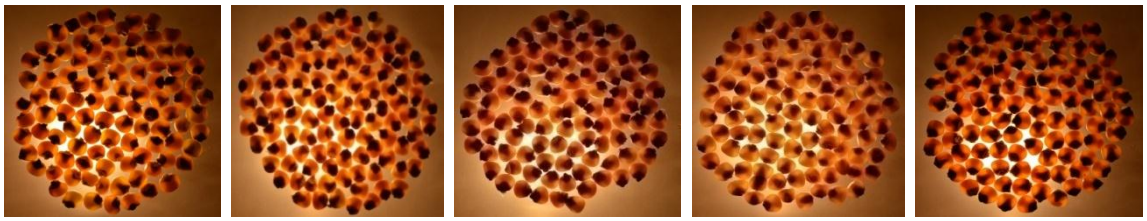


UMQ 161x373-10 Kernel score: 3 | UMQ 161x373-11 Kernel score: 3 | UMQ 161x373-12 Kernel score: 2 | UMQ 161x373-13 Kernel score: 3 | UMQ 161x373-14 Kernel score: 3

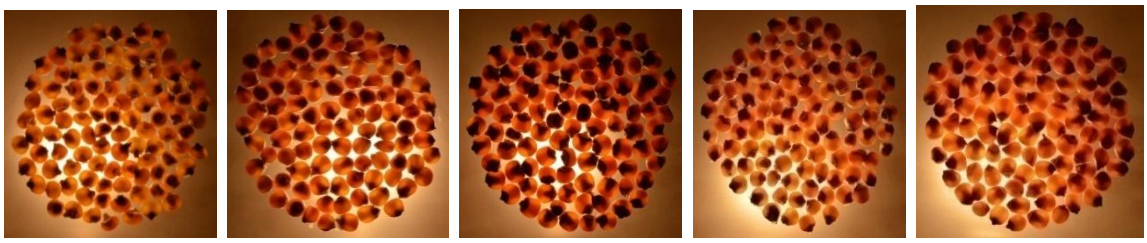


UMQ 161x373-15 Kernel score: 3 | UMQ 161x373-16 Kernel score: 3 | UMQ 161x373-17 Kernel score: 3 | UMQ 161x373-18 Kernel score: 3 | UMQ 161x373-19 Kernel score: 3

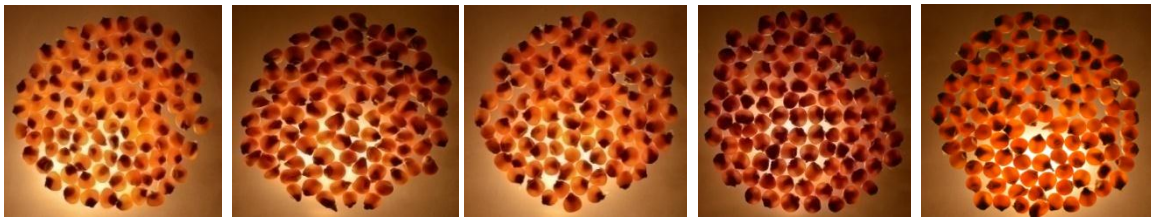
Fig. 4.1 (cntd.) Light box images and respective scores for the selected sixty lines in the $F_{3:4}$ generation



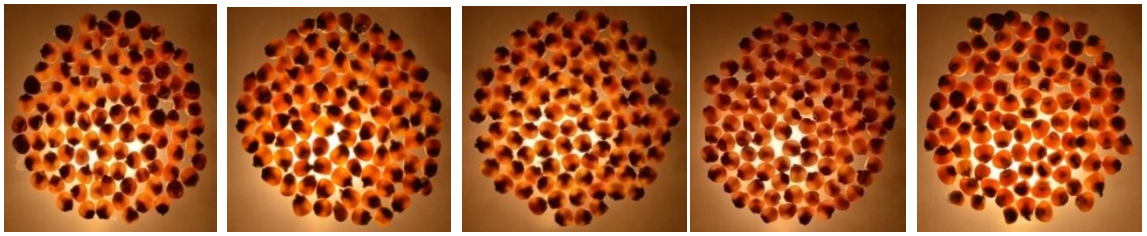
UMQ 161x373-20 Kernel score: 3 | UMQ 161x373-21 Kernel score: 3 | UMQ 161x373-22 Kernel score: 3 | UMQ 161x373-23 Kernel score: 3 | UMQ 161x373-24 Kernel score: 3



UMQ 161x373-25 Kernel score: 2 | UMQ 161x373-26 Kernel score: 3 | UMQ 161x373-27 Kernel score: 2 | MQ 161x373-28 Kernel score: 3 | UMQ 161x373-29 Kernel score: 3



UMQ 165x373-1 Kernel score: 3 | UMQ 165x373-2 Kernel score: 3 | UMQ 165x373-3 Kernel score: 3 | UMQ 165x373-4 Kernel score: 3 | UMQ 165x373-5 Kernel score: 2



UMQ 165x373-6 Kernel score: 3 | UMQ 165x373-7 Kernel score: 3 | UMQ 165x373-8 Kernel score: 3 | UMQ 165x373-9 Kernel score: 2 | UMQ 341x373-1 Kernel score: 3

Fig. 4.1 (cntd.) Light box images and respective scores for the selected sixty lines in the $F_{3:4}$ generation

4.2 Tryptophan content estimation of selected $F_{3:4}$ families

The tryptophan content in maize kernels for the selected sixty QPM lines estimated using Hopkins Cole reaction ranged from a minimum of 0.020 % to a maximum of 0.094 % (Fig. 4.2) while values ranged from 0.054 % to 0.020 % for the five normal open pollinated maize tested. It was observed that twenty two sib lines from fifteen QPM families recorded tryptophan content above the acceptable 0.070% with an average of 0.086 % (Fig. 4.3). These selected lines had recorded 5 % higher tryptophan content compared to the previous generation base population mean values (Fig. 4.4). For the remaining thirty eight lines, mean values for percent tryptophan varied from 0.063 to as low as 0.020. Base population value for % tryptophan in the F_2 population and $F_{2:3}$ sib families from which these lines have been derived were 0.044 and 0.081 respectively.

4.3 Genetic Gain (R), Selection Differential (S) and Realized Heritability (R/S) in $F_{3:4}$ families for tryptophan content

Increase in mean values for average % tryptophan content of the selected twenty two lines in $F_{3:4}$ were observed compared to the previous generations (Fig 4.4). As seen from the trend line depicting the best fit, the coefficient of determination (R^2) was 83%. For individual lines, predicted genetic gain compared to the average of the previous generation was varied (Fig. 4.5). Eighteen lines which recorded % tryptophan content greater than 0.081 recorded positive genetic gains. The remaining six lines *viz.* UMQ 161 x 373- 24, UMQ 165 x 373- 3, UMQ 161 x 373- 16, UMQ 161 x 341- 16, UMQ 161 x 373- 26 and UMQ 161 x 341- 5 with tryptophan percent values ranging from 0.073 to 0.081 recorded negative genetic gains. Negative values were recorded for selection differential and genetic gain for UMQ 161 x 341- 16 and UMQ 161 x 373- 24. Realised heritability calculated was highest (6.40) for UMQ 161 x 341- 6 with tryptophan content of 0.087 (Table 4.3). Lines UMQ 161 x 341- 20, UMQ 161 x 373- 8, UMQ 165 x 373- 9 and UMQ 165 x 373- 1 with % tryptophan content of 0.094, 0.092, 0.092 and 0.091 respectively recorded 100% response to selection as seen from realised heritability values calculated.

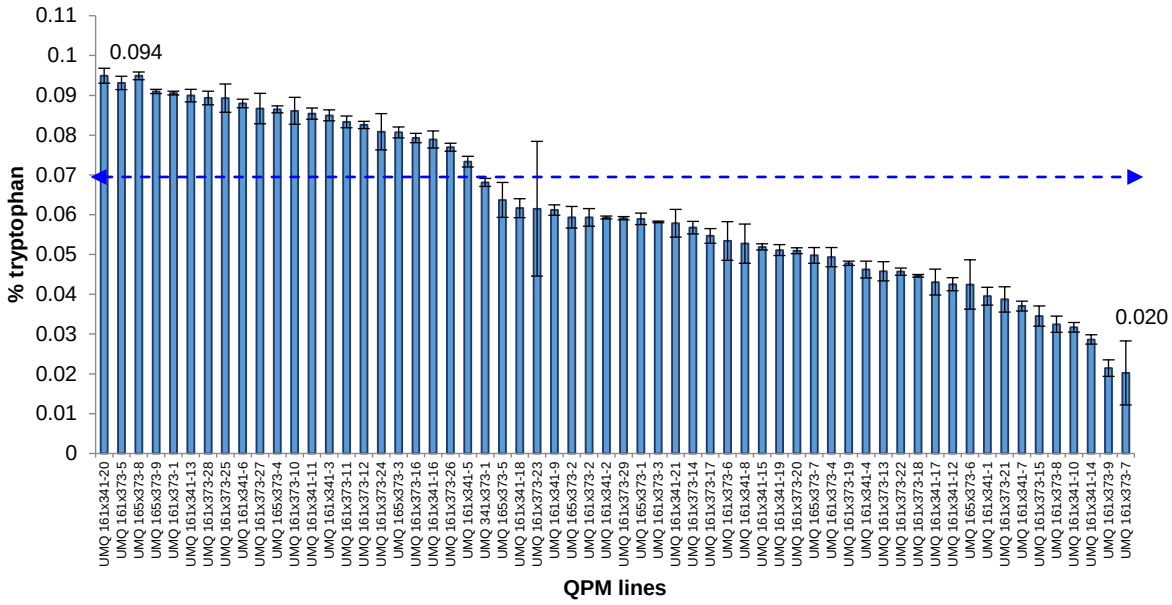


Fig. 4.2 Mean values for % tryptophan for all the sixty $F_{3:4}$ lines studied. Error bars indicate ±Standard Error. The dashed line indicates acceptable range of 0.07 % tryptophan.

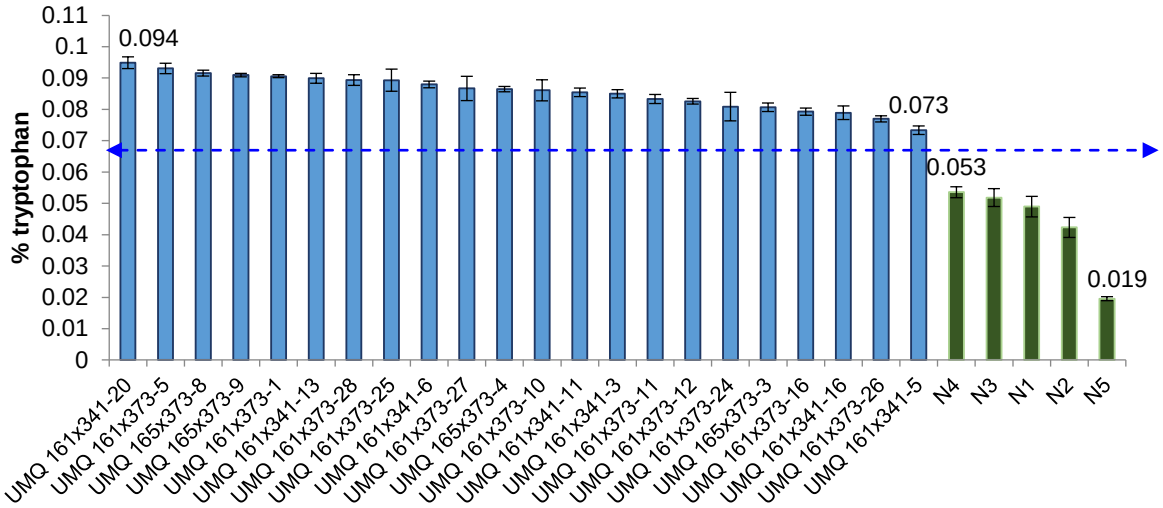


Fig. 4.3 Mean values of % tryptophan for selected twenty two $F_{3:4}$ lines compared to normal maize (N1, N2, N3, N4 and N5). The dashed line indicates acceptable range of 0.07 % tryptophan. Error bars indicate ±Standard Error.

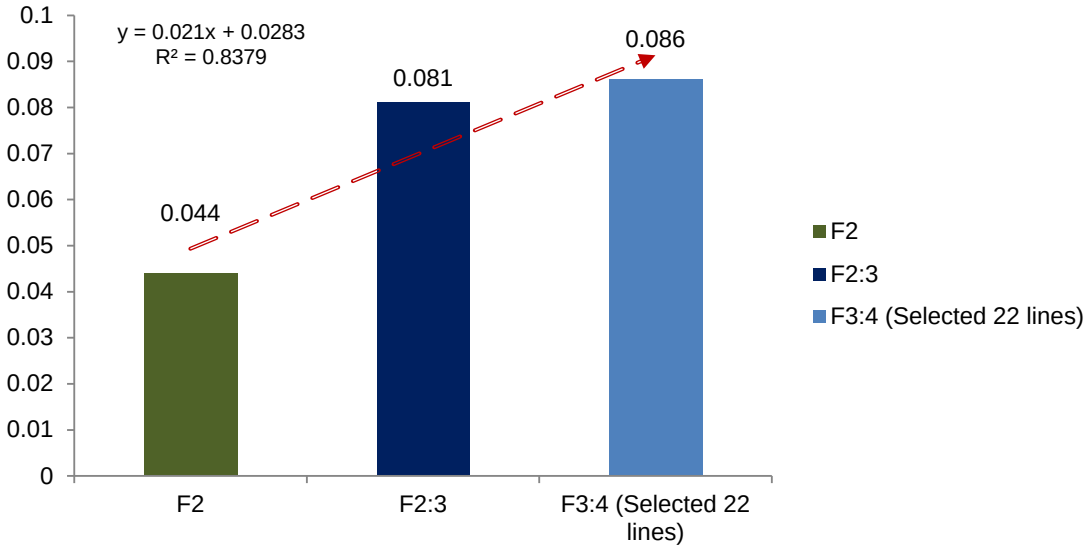


Fig. 4.4 Mean values of % tryptophan for all the selected $F_{3:4}$ lines compared to previous generations

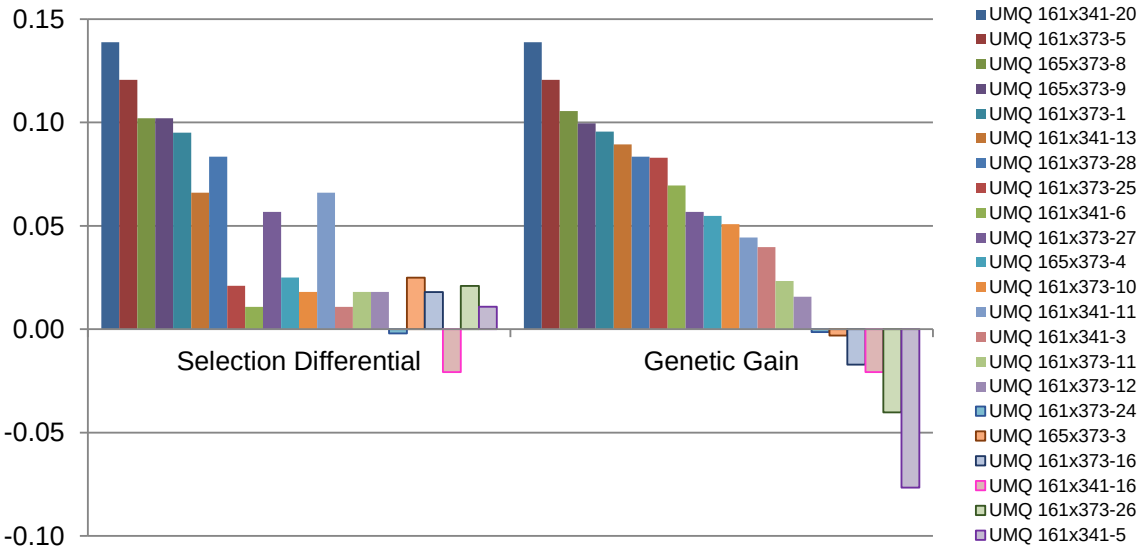


Fig. 4.5 Selection differential (S) and Genetic gain of selected $F_{3:4}$ twenty two lines for % tryptophan.

Table 4.3 Realized Heritability (R/S) for % tryptophan in the selected twenty QPM lines

Sl. No	Selected lines	% tryptophan	Realised Heritability
1	UMQ 161x341-6	0.087	6.40
2	UMQ 161x373-25	0.089	3.95
3	UMQ 161x341-3	0.085	3.66
4	UMQ 161x373-10	0.086	2.83
5	UMQ 165x373-4	0.086	2.19
6	UMQ 161x373-11	0.083	1.29
7	UMQ 165x373-9	0.092	1.08
8	UMQ 165x373-8	0.092	1.03
9	UMQ 161x373-1	0.091	1.01
10	UMQ 161x341-16	0.073	1.00
11	UMQ 161x341-20	0.094	1.00
12	UMQ 161x373-5	0.089	1.00
13	UMQ 161x373-27	0.087	1.00
14	UMQ 161x373-28	0.089	1.00
15	UMQ 161x341-13	0.090	0.92
16	UMQ 161x373-12	0.083	0.87
17	UMQ 161x373-24	0.081	0.68
18	UMQ 161x341-11	0.091	0.67
19	UMQ 165x373-3	0.081	-0.12
20	UMQ 161x373-16	0.079	-0.95
21	UMQ 161x373-26	0.077	-1.91
22	UMQ 161x341-5	0.073	-7.05
	Base population mean= 0.081		

4.4 Characterization of $F_{3:4}$ for yield, yield contributing and quality traits

Characterization of the fifteen sib families from which the twenty two selected lines were derived was done for days to 50% tasseling, days to 50% silking, Anthesis Silking Interval (ASI), plant height (cm), ear height (cm), cob length (cm), cob diameter (cm), number of kernels rows per ear, number of kernels per row, seed index (g) and yield per plant (g) (Table 4.4). ANOVA revealed significant variation at 0.05% level of significance for cob diameter and number of kernels per row. Genetic covariance for cob diameter and number of kernels per row was observed to be more than half of the respective phenotypic covariance which was reflected as 50 and 54% broad sense heritability respectively. Genetic covariance recorded for plant and ear height was also substantial and translated into 39% and 29% heritability broad sense for the respective traits. Wide variation was observed for the remaining traits studied as a result of which heritability estimates were also very low. The most variable trait recorded was for yield/plant which was calculated as total kernel weight/plant. For the selected twenty two lines, total kernel weight/plant was found to vary from 88 g to 28 g in the families studied while seed index ranged from 40.1 g to 20.4 g (Fig. 4.6).

For the qualitative traits studied (Table 4.5) it was observed that kernel colour varied from yellow to white and brown but was yellow for majority of the lines under study. Kernel texture semi flint in fifteen of the twenty two lines studied and flint for the remaining seven lines. Kernel row arrangement was irregular in ten of the twenty two lines studied and straight in the remaining twelve. Most of the lines under study showed presence of anthocyanin in tassel, ear and silk. The attitude of lateral tassel branches and leaf angle varied between curved and fully curved in the lines studied.

Table 4.4 Yield and yield contributing traits

Sl. No.	Character	MEAN ±SEM	MSS	PCV	GCV	RANGE	h_{bs}
1.	Days to 50% tasseling	68 days ± 1.73	8.53	3.95	1.65	64-76	0.17
2.	Days to 50% silking	75 days ± 1.77	7.90	3.56	1.22	69-83	0.12
3.	Anthesis Silking Interval	6 days± 0.53	0.74	13.86	5.14	4-8	0.14
4.	Plant height (cm)	161 cm ± 7.43	167.13	8.30	5.15	143.50-188.50	0.39
5.	Ear height (cm)	73 cm ± 5.39	30.69	12.35	6.59	57.30-87.30	0.28
6.	Cob length (cm)	12 cm ± 1.83	8.09	22.21	6.74	8.17-17.05	0.09
7.	Cob Diameter (cm)	4 cm ± 0.22	0.28*	11.05	7.81	2.92-5.12	0.50
8.	No. of kernel rows per ear	13 ± 0.99	2.45	11.53	3.92	10-16	0.12
9.	No. of kernels per row	13 ± 1.68	3.36*	26.75	19.67	2-21	0.54
10.	Seed index (g)	25 g ± 2.86	31.37	19.44	10.91	14.20-40.30	0.32
11.	Yield per plant (g)	39 g ± 9.68	230.03	36.33	12.75	4.60-85.98	0.12

Standard Error Mean (SEM), Mean Sum Squares (MSS), Phenotypic Covariance (PCV), Genotypic Covariance (GCV), Broad sense heritability (h_{bs}), Genetic Advance (GA).

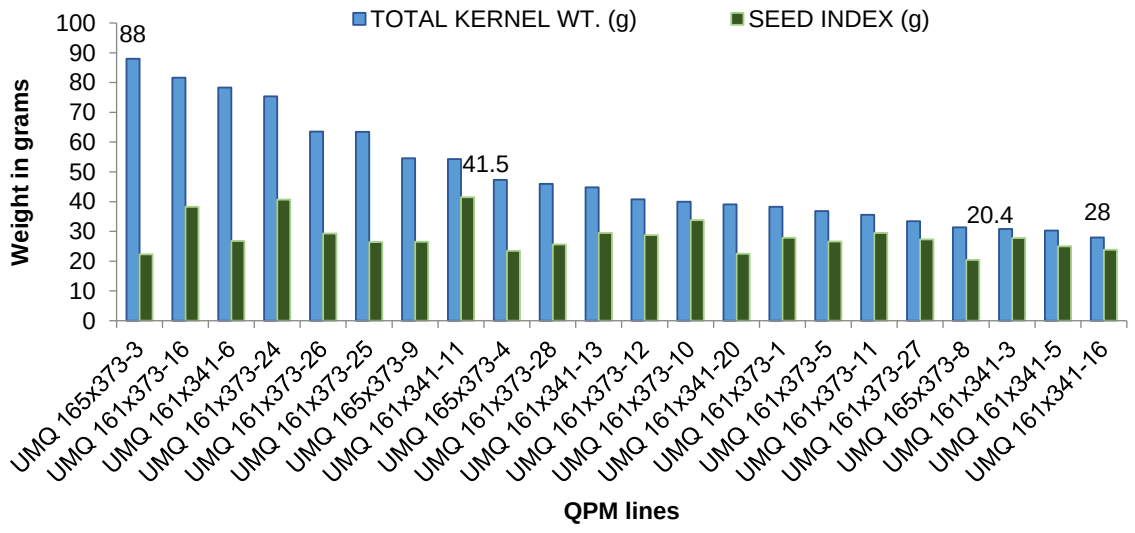


Fig. 4.6 The yield/plant (g) and seed index (g) for the selected twenty two lines studied

Table 4.5 Qualitative attributes recorded for selected lines

Sl. No	Lines	KC	KT	KRA	TA	EA	ALB	LA	SH	SA
1	UMQ 161x341-3	Yellow	Flint	Irregular	Present	Absent	Curved	Curved	Present	Present
2	UMQ 161x341-5	Yellow	Semi flint	Irregular	Absent	Present	Fully curved	Curved	Present	Present
3	UMQ 161x341-6	Yellow	Semi flint	Irregular	Present	Present	Curved	Curved	Present	Absent
4	UMQ 161x341-11	White	Semi flint	Straight	Present	Present	Curved	Fully curved	Present	Present
5	UMQ 161x341-13	White	Semi flint	Straight	Present	Present	Curved	Curved	Present	Present
6	UMQ 161x341-16	White	Semi flint	Irregular	Present	Present	Curved	Curved	Present	Present
7	UMQ 161x341-20	Yellow	Flint	Straight	Absent	Absent	Curved	Curved	Present	Present
8	UMQ 161x373-1	Yellow	Flint	Irregular	Present	Present	Fully curved	Fully curved	Present	Present
9	UMQ 161x373-5	Yellow	Flint	Irregular	Present	Present	Fully curved	Curved	Present	Present
10	UMQ 161x373-10	White	Flint	Straight	Absent	Present	Curved	Curved	Present	Present
11	UMQ 161x373-11	Yellow	Semi flint	Straight	Present	Present	Curved	Curved	Present	Present
12	UMQ 161x373-12	Yellow	Flint	Straight	Present	Present	Curved	Curved	Present	Present
13	UMQ 161x373-16	Brown	Semi flint	Straight	Present	Present	Curved	Curved	Present	Absent
14	UMQ 161x373-24	Yellow	Semi flint	Irregular	Present	Present	Curved	Fully curved	Present	Present
15	UMQ 161x373-25	Yellow	Flint	Irregular	Present	Absent	Curved	Curved	Present	Absent
16	UMQ 161x373-26	Yellow	Semi flint	Straight	Present	Absent	Curved	Curved	Present	Absent
17	UMQ 161x373-27	Yellow	Semi flint	Irregular	Present	Present	Curved	Curved	Present	Absent
18	UMQ 161x373-28	Yellow	Semi flint	Irregular	Present	Present	Curved	Fully curved	Present	Absent
19	UMQ 165x373-3	Yellow	Semi flint	Straight	Present	Present	Curved	Fully curved	Present	Absent
20	UMQ 165x373-4	Brown	Semi flint	Straight	Present	Present	Curved	Curved	Present	Absent
21	UMQ 165x373-8	Yellow	Semi flint	Straight	Present	Present	Curved	Fully curved	Present	Absent
22	UMQ 165x373-9	Yellow	Seimi flint	Straight	Present	Present	Curved	Curved	Present	Present

Kernel Colour (KC), Kernel Texture (KT), Kernel Row Arrangement (KRA), Tassel Anthocyanin (TA), Silk Anthocyanin (EA), Atitude of Lateral Branches (ALB), Leaf Angle (LA), Stalk Hair (SH), Stalk Anthocyanin (SA)

4.5 Molecular characterization of selected $F_{3:4}$ QPM sib families

A total of 20 *o2* specific and associated SSR markers from chromosomal bins 1.01, 1.08, 2.07, 3.04, 7.00, 7.01, 7.02 and 9.05 (Table 4.6) were used to confirm the identity of QPM lines as distinct from normal maize genotypes. Three of twenty markers used in the study *phi057*, *phi112* and *umc1066* were gene specific markers from bin 7.01 while the remaining seventeen markers were associated with polygenic QPM modifiers in bin 7.02 or with modifiers for vitreous endosperm distributed in bins1.01, 1.08, 2.07, 3.04, and 9.05 (Table 4.6). A total of twenty nine lines were characterized using these 20 markers where lines 1-22 had recorded % tryptophan ranging from 0.094 to 0.073, lines 23-27 recorded % tryptophan ranging from 0.042 to 0.061 and lines 28 and 29 were normal maize genotypes with % tryptophan of 0.035 and 0.019 respectively. Using allelic data, all twenty nine lines studied could be clustered into three distinct classes where QPM lines with high % tryptophan grouped into Clusters I and II. Percent tryptophan for lines grouped in Cluster I varied from 0.091 to 0.073 with an average of 0.084. In Cluster II, barring L25 (UMQ 161x341-12) with a % tryptophan value of 0.043, the average % tryptophan for the remaining lines were 0.087. For lines which grouped in cluster III, with the exception of L14 (UMQ 161x 373 -24) with % tryptophan value 0.080, the average % tryptophan value was 0.036. The tree distances (Table 4.7) calculated using Unweighted Neighbour Joining for the QPM normal maize lines was minimum (0.10) between lines 4 and 13 and maximum between 18 and 28 (0.47).

Distinct polymorphism was observed for co dominant gene specific markers *phi057 and umc1066* between the QPM lines 1 to 22 and Normal Maize with a few exceptions where the lines were heterozygous (Fig. 4.8). For the dominant SSR marker *phi112*, excepting lines 9, 17, 18, 23, 24 there was no amplification in the QPM lines was observed. Based on the banding pattern of these three markers most of these QPM lines could be clearly distinguished form the Normal Maize genotypes. Polymorphism for normal maize was also observed for the remaining SSR markers used. Polymorphism Information Content values of these 20 markers ranged from 0.19 to 0.65 with an average of 0.44 while percent heterozygosity ranged from a minimum of 0.04 to a maximum of 0.45 and 100 % homozygosity observed in three SSR markers *viz. bnlg2160, phi034, umc1831*. The number of alleles varied from 2 to 3.

Table 4.6 Polymorphism among 20 *o2* associated markers used in this study

Sl. No.	Markers	Associated trait	Bin No.	PIC	% Heterozygosity	No. of alleles
1.	*umc1695*	Associated with *o2* gene	7.00	0.51	0.14	2
2.	*bnlg2132*	Flanking marker for *o2* gene	7.00	0.30	0.07	3
3.	*phi057*	*o2* specific gene	7.01	0.44	0.18	3
4.	*phi112*	*o2* specific gene	7.01	0.50	0.25	2
5.	*umc1066*	*o2* specific gene	7.01	0.42	0.28	3
6.	*umc1929*	Vitreous endosperm associated with 27 kDa γ zein	7.02	0.53	0.45	3
7.	*umc1393*	Vitreous endosperm associated with 27 kDa γ zein	7.02	0.46	0.40	2
8.	*umc1036*	Flanking marker for 27 kDa γ zein	7.02	0.32	0.16	3
9.	*umc1978*	Vitreous endosperm associated with 27 kDa γ zein	7.02	0.49	0.19	2
10.	*bnlg1200*	Associated with *o2* gene	7.01	0.50	0.17	3
11.	*bnlg2160*	Associated with *o2* modifier loci	7.01	0.38	0.00	3
12.	*phi034*	Associated with *o2* modifier loci	7.02	0.50	0.00	3
13.	*umc1831*	Associated with *o2* modifier loci	7.02	0.19	0.00	3
14.	*umc1016*	Vitreous endosperm associated with 27 kDa γ zein	7.02	0.62	0.44	3
15.	*bnlg1164*	Flanking with 27 kDa γ zein	7.02	0.48	0.13	2
16.	*bnlg1179*	Modifiers for vitreous endosperm and *o2* gene	1.01	0.49	0.10	2
17.	*bnlg1643*	Modifiers for vitreous endosperm and *o2* gene	1.08	0.36	0.04	2
18.	*bnlg1633*	Modifiers for vitreous endosperm and *o2* gene	2.07	0.48	0.04	2
19.	*bmc2136*	Modifiers for vitreous endosperm and *o2* gene	3.04	0.65	0.43	3
20.	*umc1357*	Modifiers for vitreous endosperm and *o2* gene	9.05	0.27	0.13	2

Table 4.7 Dissimilarity matrix using Unweighted Neighbour Joining for QPM normal maize lines based on allelic data

LINES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
2	0.15																											
3	0.17	0.14																										
4	0.18	0.22	0.25																									
5	0.24	0.28	0.31	0.27																								
6	0.19	0.24	0.26	0.22	0.18																							
7	0.24	0.28	0.30	0.18	0.33	0.28																						
8	0.21	0.26	0.28	0.20	0.31	0.26	0.26																					
9	0.22	0.26	0.29	0.21	0.31	0.27	0.27	0.25																				
10	0.18	0.22	0.25	0.21	0.21	0.16	0.26	0.24	0.25																			
11	0.19	0.24	0.26	0.18	0.29	0.24	0.24	0.18	0.23	0.22																		
12	0.23	0.28	0.30	0.26	0.26	0.22	0.32	0.30	0.30	0.17	0.28																	
13	0.15	0.20	0.22	**0.10**	0.25	0.20	0.12	0.18	0.19	0.18	0.16	0.24																
14	0.17	0.21	0.24	0.19	0.26	0.21	0.25	0.23	0.23	0.20	0.20	0.25	0.17															
15	0.24	0.28	0.30	0.27	0.31	0.26	0.32	0.30	0.31	0.24	0.28	0.30	0.24	0.26														
16	0.16	0.20	0.23	0.19	0.19	0.14	0.25	0.22	0.23	0.11	0.20	0.16	0.17	0.18	0.22													
17	0.22	0.27	0.29	0.17	0.32	0.27	0.23	0.24	0.26	0.25	0.22	0.31	0.15	0.23	0.31	0.23												
18	0.25	0.30	0.32	0.28	0.32	0.27	0.34	0.32	0.32	0.26	0.30	0.31	0.26	0.27	0.23	0.24	0.33											
19	0.19	0.23	0.25	0.26	0.33	0.28	0.32	0.30	0.30	0.26	0.28	0.32	0.24	0.25	0.32	0.24	0.31	0.34										
20	0.24	0.28	0.30	0.23	0.33	0.28	0.29	0.26	0.23	0.26	0.24	0.32	0.20	0.25	0.32	0.25	0.27	0.34	0.32									
21	0.14	0.18	0.20	0.21	0.28	0.23	0.27	0.25	0.26	0.21	0.23	0.27	0.19	0.20	0.27	0.19	0.26	0.29	0.18	0.27								
22	0.21	0.26	0.28	0.24	0.29	0.24	0.30	0.28	0.29	0.22	0.26	0.28	0.22	0.23	0.23	0.20	0.29	0.24	0.30	0.30	0.25							
23	0.19	0.23	0.26	0.21	0.28	0.24	0.27	0.25	0.26	0.22	0.23	0.28	0.19	0.19	0.28	0.20	0.26	0.30	0.28	0.27	0.23	0.26						
24	0.23	0.27	0.30	0.25	0.32	0.27	0.31	0.29	0.29	0.26	0.26	0.31	0.23	0.23	0.31	0.24	0.29	0.33	0.31	0.31	0.26	0.29	0.17					
25	0.22	0.27	0.29	0.22	0.32	0.27	0.27	0.25	0.22	0.25	0.23	0.31	0.19	0.24	0.31	0.23	0.26	0.33	0.31	0.21	0.26	0.29	0.26	0.30				
26	0.20	0.25	0.27	0.22	0.30	0.25	0.28	0.26	0.27	0.23	0.24	0.29	0.20	0.19	0.29	0.21	0.27	0.31	0.29	0.28	0.24	0.27	0.23	0.26	0.27			
27	0.20	0.24	0.27	0.22	0.29	0.24	0.28	0.26	0.26	0.23	0.24	0.28	0.20	0.19	0.29	0.21	0.26	0.30	0.28	0.28	0.24	0.27	0.22	0.26	0.27	0.17		
28	0.36	0.41	0.43	0.39	0.46	0.41	0.44	0.42	0.43	0.39	0.40	0.45	0.36	0.36	0.45	0.37	0.43	**0.47**	0.45	0.44	0.40	0.43	0.34	0.37	0.43	0.40	0.40	
29	0.30	0.35	0.37	0.33	0.40	0.35	0.38	0.36	0.37	0.33	0.34	0.39	0.30	0.30	0.39	0.31	0.37	0.41	0.39	0.38	0.34	0.37	0.28	0.31	0.37	0.34	0.34	0.39

1: UMQ 161x341-3, 2: UMQ 161x341-5; 3: UMQ 161x341-6, 4: UMQ 161x341-11, 5: UMQ 161x341-13, 6: UMQ 161x341-16, 7: UMQ 161x341-20, 8: UMQ 161x373-1, 9: UMQ 161x373-5, 10: UMQ 61x373-10, 11: UMQ 161x373-11, 12: UMQ 161x373-12, 13: UMQ 161x373-16, 14: UMQ 161x373-24, 15: UMQ 161x373-25, 16: UMQ 161x373-26, 17: UMQ 161x373-27, 18: UMQ 161x373-28, 19: UMQ 165x373-3, 20: UMQ 165x373-4, 21: UMQ 165x373-8, 22: UMQ 165x373-9, 23: UMQ 161x341-1, 24: UMQ 161x341-4, 25: UMQ 161x341-12, 26: UMQ 161x341-17, 27:UMQ 161x373-29, 28: Normal Maize, 29: Normal Maize

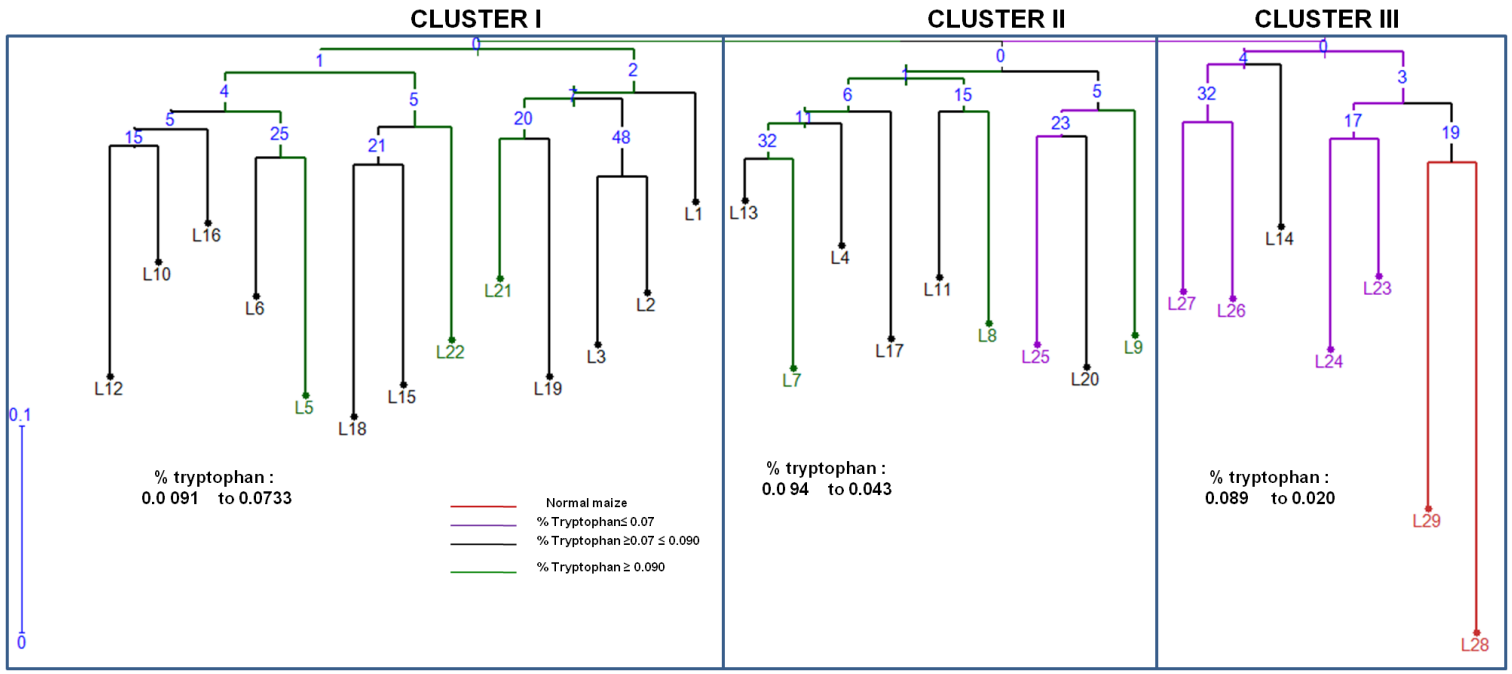
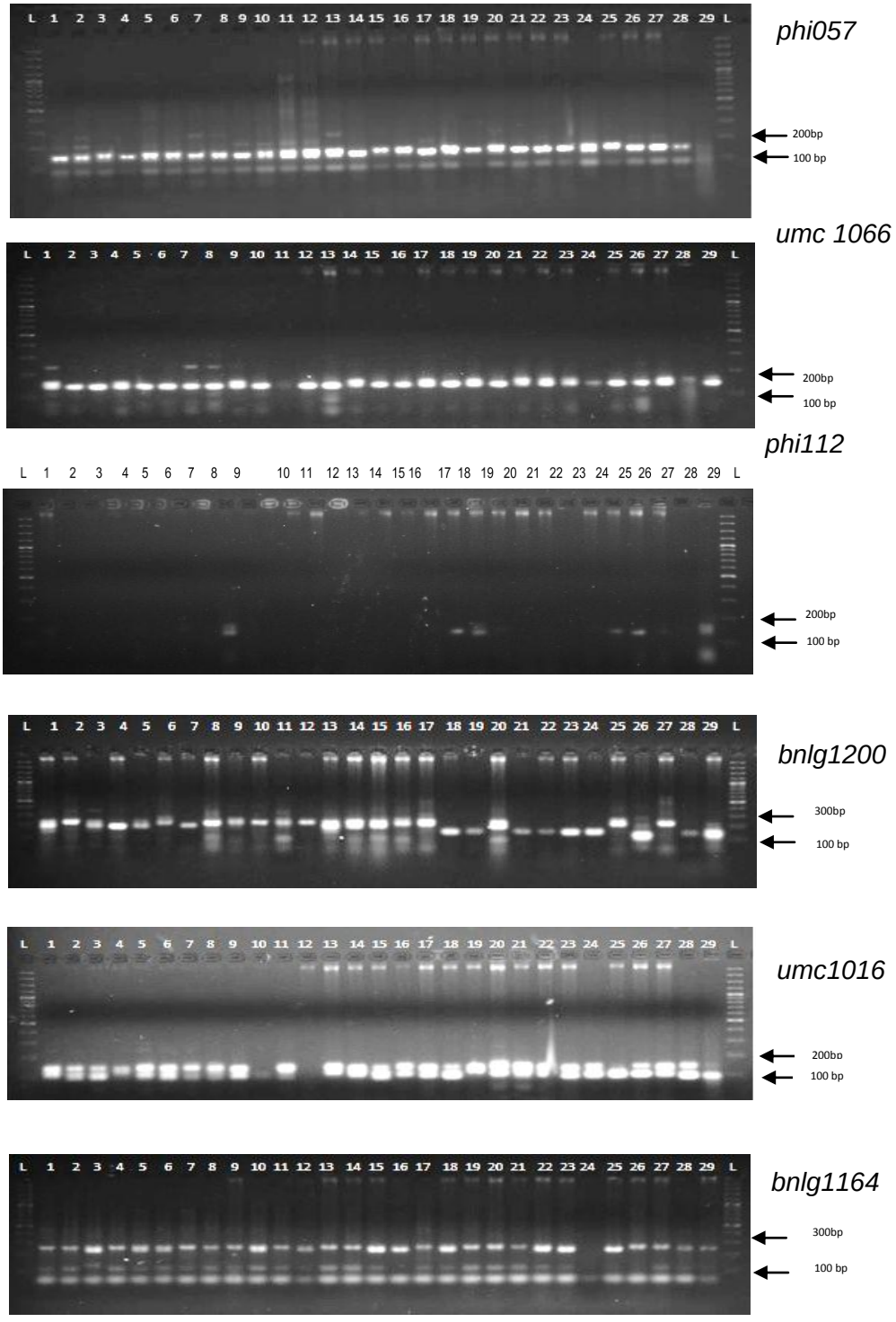


Fig. 4.7 Dendrogram depicting genetic relationships among the twenty nine inbred lines including QPM and normal maize lines used for SSR analysis

1: UMQ 161x341-3, 2: UMQ 161x341-5; 3: UMQ 161x341-6, 4: UMQ 161x341-11, 5: UMQ 161x341-13, 6: UMQ 161x341-16, 7: UMQ 161x341-20, 8: UMQ 161x373-1, 9: UMQ 161x373-5, 10: UMQ 61x373-10, 11: UMQ 161x373-11, 12: UMQ 161x373-12, 13: UMQ 161x373-16, 14: UMQ 161x373-24, 15: UMQ 161x373-25, 16: UMQ 161x373-26, 17: UMQ 161x373-27, 18: UMQ 161x373-28, 19: UMQ 165x373-3, 20: UMQ 165x373-4, 21: UMQ 165x373-8, 22: UMQ 165x373-9, 23: UMQ 161x341-1, 24: UMQ 161x341-4, 25: UMQ 161x341-12, 26: UMQ 161x341-17, 27:UMQ 161x373-29, 28: Normal Maize, 29: Normal Maize



1: UMQ 161x341-3, 2: UMQ 161x341-5; 3: UMQ 161x341-6, 4: UMQ 161x341-11, 5: UMQ 161x341-13, 6: UMQ 161x341-16, 7: UMQ 161x341-20, 8: UMQ 161x373-1, 9: UMQ 161x373-5, 10: UMQ 61x373-10, 11: UMQ 161x373-11, 12: UMQ 161x373-12, 13: UMQ 161x373-16, 14: UMQ 161x373-24, 15: UMQ 161x373-25, 16: UMQ 161x373-26, 17: UMQ 161x373-27, 18: UMQ 161x373-28, 19: UMQ 165x373-3, 20: UMQ 165x373-4, 21: UMQ 165x373-8, 22: UMQ 165x373-9, 23: UMQ 161x341-1, 24: UMQ 161x341-4, 25: UMQ 161x341-12, 26: UMQ 161x341-17, 27:UMQ 161x373-29, 28: Normal Maize, 29: Normal Maize, L: Molecular Ladder

Fig. 4.8 Gel electrophoresis showing polymorphic patterns for o2 specific SSR markers *phi057, umc1066, phi112* associated modifiers *bnlg1200, umc1016, bnlg1164* for the twenty nine lines/genotypes studied.

Chapter 5

Discussion

North East India relies primarily on animal protein to supplement the diet and successful QPM cultivation would provide both health and economic benefits to the region predominantly under acidic soil conditions. QPM are known to posses two-to-threefold increased levels of lysine and tryptophan in comparison with normal maize (Babu and Prasanna, 2014). To gain wider acceptance it is therefore imperative that QPM hybrids tolerant to acidic soil conditions are popularised along with better yield. In the present study, $F_{3:4}$ generation were raised from F_2 populations of CML 161 X CML 165, CML 161 X V 390, CML 161 X V 341, CML 161 X V 373, CML 165 X V 341, CML 165 X V 373, V 390 X V 341 and V 341 X V 373 (Backiyalakshmi, 2016) to select for QPM lines with superior tryptophan content. These six F_2 populations had been sib mated within each population in the previous year (Khati, 2018) and segregants with kernel modification score 2/3 and high seed index were advanced to raise the $F_{3:4}$ sib families.

Phenotypic screening of the six hundred and eighty one lines for endosperm modification using light box method identified four hundred and forty one lines (64%) with kernel modification score of 2-3 indicating less than 25% opacity. Since it was practically not feasible to forward all the four hundred and forty one families, using test weight as a general indicator of seed vigour, sixty lines with the highest test weight were selected for estimation of % tryptophan content as per Hopkins Cole reaction. Lysine and tryptophan values are highly correlated with the value of lysine being four times that of tryptophan and therefore tryptophan can be used as a single parameter for evaluating the nutritional protein quality of the protein (Vivek *et al.*, 2008). For the current study, considerable variation was observed for % tryptophan content ranging from 0.020 % to 0.094 % in the segregants studied. Twenty two sibbed lines from fifteen QPM families recorded tryptophan content above the acceptable 0.070% with an average of 0.086 % recording a 5% mean increase over the previous generation. High realized heritability values recorded for most of these lines indicate a genetic basis in transmission of % tryptophan. Values for the five normal open pollinated maize tested ranged from 0.019 % to 0.054 %. Segregating generations of QPM are generally associated with significant variation for % tryptophan

among lines because of the polygenic nature of modifier genes which are responsible for phenotypic expression of the trait.

Babu *et al.* (2005) while studying the feasibility of combining high protein quality and hard endosperm characters in early maturing normal maize inbreds observed several classes of kernel modification in each population of BC_2F_2 for harvested ears that were homozygous for *o2* gene with varying proportions of kernel opaqueness. Tryptophan in protein for these BC_2F_2 families ranged from 0.78 to 0.94%. Tufchi *et al.* (2015) studying the effect of expression of *o2* gene on tryptophan concentration noted significant variance in the $BC_2F_{2:3}$ progenies ranging from a minimum of 0.046 to a maximum of 0.082 %. Differential gene expression of the for % tryptophan content can also be because the segregants may possess o2 alleles with two different haplotypes contributed by the parental inbreds as reported by Pandey *et al.* (2018). They had reported the presence of five different alleles of the *o2* gene in a panel of forty six QPM inbreds which can influence the expression of tryptophan content from very high to well below acceptable standards that define QPM.

Distinct polymorphism was observed between the normal and QPM inbred lines with respect to all the three gene specific SSR markers *phi057*, *phi112* and *umc1066* validating the identity of the lines under study. Polymorphism for *phi112* was dominant as reflected by null allele in all but four QPM lines. Absence of amplification for *phi112* in QPM is a purity test for maintenance QPM lines (Babu *et al.*, 2005). However this marker is not fool proof as diverse backgrounds of o2o2 germplasm also present a band with this marker (Vivek *et al.*, 2008). The presence of the band in QPM lines when using *phi112* can also indicate possible pollen contamination from the normal maize during seed maintenance (Ignjatovic-Micic *et al.*, 2009). SSR markers for *phi057* and *umc1066* were co-dominant and could clearly distinguish QPM and non QPM lines under study with a few exceptions. Similarly most of the SSR markers associated with endosperm hardness or enhancement of tryptophan also showed distinct polymorphism with the normal maize lines used in the study. The allelic data when used for cluster analysis could successfully group the tryptophan lines into three distinct classes which could be corroborated with the phenotypic expression of % tryptophan. Lines with high % tryptophan grouped into Clusters I and II, while the lines with % tryptophan below 0.070 grouped into Cluster III including the two normal maize lines under study.

Since yield is the primary objective of the breeder, it is essential that when selection is done, yield contributing traits with higher genetic contribution receive due emphasis. As pointed out by Ignjatovic-Micic *et al.* (2009) lines with lower

tryptophan content can be a good choice only if their agronomical characteristics are good while lines with higher tryptophan content can be a poor choice if having unsatisfactory agronomic characteristics. Kostadinovic *et al.* (2016) no significant correlations between biochemical and majority of phenotypic and agronomic components in QPM lines but highlighted poor seed set as a major impediment in QPM development.

Lal and Singh (2014) while studying the magnitude of genetic variability for morphological and quality traits in fifty seven QPM genotypes observed considerable action of non additive gene action for the different traits studied. In the current study, for the various yield and yield contributing traits, it was observed that significant variation was present for cob diameter and number of kernels per row reflected as 50 and 54% broad sense heritability respectively. Total kernel weight (yield / plant) for the selected twenty two lines with high % tryptophan was the most variable character ranging from 88 g to 28 g while seed index ranged from 41.5 to 20.4 g. Since higher number of kernels contribute to increased yield per plant and in our case has a genetic basis, it would be desirable to select for *o2* lines with higher kernel number for further advancement in the lines under study. It is important to focus on traits with high heritability as stable phenotypic expression of agronomical traits in *o2* homozygous individuals provides an excellent opportunity to combine the desirable agronomic traits with superior protein quality (Babu *et al.*, 2005). The objective of current study was to screen the segregating generation for lines with high tryptophan content and confirm their genetic identity for use in future breeding programme. Twenty two lines with high tryptophan value could be successfully identified. Of these, five lines UMQ 161x341-13, UMQ 161x341-20, UMQ 161x373-1, UMQ 165x373-8 and UMQ 165x373-9 with % tryptophan values ranging from 0.90 to 0.94 identified as distinct from non QPM maize can be further taken up for line development programme as the emphasis is now required to shift towards development of agronomically superior population/lines.

Chapter 6

Summary and Conclusion

QPM (Quality Protein Maize) with recessive *o2* (*opaque 2*) gene in combination with polygenic modifiers significantly influence the protein quality and endosperm modification resulting in nutritionally superior maize with higher tryptophan and lysine content. However, QPM cultivation has restricted acceptance which could be rectified if QPM hybrids with agronomic performance at par to normal hybrids were to be released. In North Eastern Hill (NEH) Region of India, soils are predominantly acidic and it is imperative that QPM hybrids tolerant to acidic soil conditions are developed along with better yield. If successful, it would provide a cost effective access to high quality protein since the diet of the people of this region is predominantly from animal protein.

A combining ability study using half diallel mating involving crosses CML 161 X CML 165, CML 161 X V 390, CML 161 X V 341, CML 161 X V 373, CML 165 X V 341, CML 165 X V 373, V 390 X V 341 and V 341 X V 373 was initiated in Kharif 2015 where parental line CML 165 and CML 165 x V 341 showed great promise for both tryptophan content and grain yield. The six F_2 populations generated were advanced to the F_3 generation based on kernel modification score and % tryptophan content. The current study focused on raising of the forwarded F_4 generation to identify segregants with superior tryptophan content based on phenotypic screening using light box method and biochemical estimation for % tryptophan and selection of homozygous lines using reported SSR markers for *o2* and *o2* modifiers.

Phenotypic screening of the six hundred and eighty one lines for endosperm modification using light box method identified four hundred and forty one lines (64%) with kernel modification score of 2-3 indicating less than 25% opacity. Since it was practically not feasible to forward all the four hundred and forty one families, using test weight as a general indicator of seed vigour, sixty lines with the highest test weight were selected for estimation of % tryptophan content as per Hopkins Cole reaction.

Considerable variation was observed for % tryptophan content ranging from 0.020 % to 0.094 % in the segregants studied. Twenty two sibbed lines from fifteen QPM families recorded tryptophan content above the acceptable 0.070% with an average of 0.086 % recording a 5 % mean increase over the previous generation.

High realized heritability values recorded for most of these lines indicate a genetic basis in transmission of % tryptophan. Values for the five normal open pollinated maize tested ranged from 0.019 % to 0.054 %. Distinct polymorphism was observed between the normal and QPM inbred lines with respect to all the three gene specific SSR markers *phi057*, *phi112* and *umc1066* validating the identity of the lines under study. Similarly most of the SSR markers associated with endosperm hardness or enhancement of tryptophan also showed distinct polymorphism with the normal maize lines used in the study. The allelic data when used for cluster analysis could successfully group the tryptophan lines into three distinct classes. In Clusters I and II, were the lines with high % tryptophan while in Cluster III were lines with % tryptophan values below 0.070 including the two normal maize lines used for comparison.

For the twenty two selected lines with high tryptophan content, total kernel weight (yield / plant) was the most variable character ranging from 88 g to 28 g while seed index ranged from 41.5 to 20.4g. Significant variation was present for cob diameter and number of kernels per row reflected as 50 and 54% broad sense heritability respectively. Of the twenty two lines, five lines UMQ 161x341-13, UMQ 161x341-20, UMQ 161x373-1, UMQ 165x373-8 and UMQ 165x373-9 with % tryptophan values ranging from 0.90 to 0.94 identified as distinct from non QPM maize can be further taken up for line development programme as the emphasis is now required to shift towards development of agronomically superior population/lines.

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Appendix

Appendix 1 Light box score and seed index (g) values of selected lines for growing $F_{3:4}$ generation

Sl. No	Selected lines for $F_{3:4}$ generation	Light box score	Seed Index (g)
1.	V341xV373-60X75-8X9	3	37.6
2.	V341xV373-60X75-10X9	2	33.8
3.	V341xV373-68X14-6X8	3	29
4.	V341xV373-60X75-2X4	3	27.7
5.	CML165xV373-60X59-5X7	32.7	3
6.	CML165xV373-56X59-9X8	30.8	3
7.	CML165xV373-10X1-5X2	30.2	3
8.	CML165xV373-2X3-2X3	30	3
9.	CML165xV373-2X3-7X6	29.3	3
10.	CML165xV373-56X59-1X6	33.8	3
11.	CML165xV373-60X59-8X7	29.5	2
12.	CML165xV373-56X59-2X5	39.2	2
13.	CML165xV373-67X45-9X8	37	3
14.	CML161xV373-48X72-4X7	47.8	3
15.	CML161xV373-96X98-3X1	43.2	3
16.	CML161xV373-76X82-9X10	41.5	3
17.	CML161xV373-47X43-2X1	40.3	3
18.	CML161xV373-32X89-6X7	40	2
19.	CML161xV373-73X10-5X4	37.8	3
20.	CML161xV373-73X10-5X3	37.2	3
21.	CML161xV373-73X10-9X3	35.7	3
22.	CML161xV373-8X37-4X1	33.4	3
23.	CML161xV373-32X89-3X2	33	2
24.	CML161xV373-73X10-8X2	32.1	3
25.	CML161xV373-52X58-5X2	32	3
26.	CML161xV373-69X87-5X6	31.5	3

27.	CML161xV373-33X47-5X2	30.2	3
28.	CML161xV373-58X78-2X4	30	3
29.	CML161xV373-96X98-5X7	29.8	3
30.	CML161xV373-73X10-6X7	29.5	3
31.	CML161xV373-52X58-9X7	29.4	3
32.	CML161xV373-93X98-6X2	29.3	3
33.	CML161xV373-47X43-10X5	29	3
34.	CML161xV373-52X58-6X8	29	3
35.	CML161xV373-69X87-6X9	28.8	2
36.	CML161xV373-77X93-6X5	28.8	3
37.	CML161xV373-41X46-3X2	47.8	3
38.	CML161xV373-58X78-3X6	33.7	3
39.	CML161xV373-58X78-8X5	33.5	3
40.	CML161xV373-92X57-8X2	33.2	3
41.	CML161xV373-76X82-1X2	32.5	3
42.	CML161xV373-72X21-5X2	31.5	3
43.	CML161xV373-58X78-5X6	30.8	3
44.	CML161xV373-66X106-6X9	28.4	3
45.	CML161xV373-32X89-10X7	28.3	3
46.	CML161xV373-87X113-8X9	38.3	3
47.	CML161xV373 72X21-9X2	35.5	3
48.	CML161xV373-82X76-8X9	31.8	3
49.	CML161xV373-82X76-5X7	28.8	2
50.	CML161xV341-75X73-7X8	30.5	3
51.	CML161xV341-71X60-9X2	30.3	2
52.	CML161xV341-51X55-9X8	30.2	3
53.	CML161xV341-64X54-2X5	29.2	3
54.	CML161xV341-96X88-6X5	28.5	2
55.	CML161xV341-61X66-5X4	28.2	2
56.	CML161xV341-61X66-7X1	28.1	2

57.	CML161xV341-96X88-3X4	36	3
58.	CML161xV341-71X60-4X6	34.4	3
59.	CML161xV341-75X73-5X7	32.2	2
60.	CML161xV341-57X65-6X8	29.7	3