PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF HIGH LYSINE

MAIZE

A Dissertation

by

SANDEEP BHATNAGAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Plant Breeding

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December 2004

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ABSTRACT

Phenotypic and Genotypic Characterization of High Lysine Maize.

(December 2004)

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Quality Protein Maize (QPM) with the mutant gene *opaque-2* (*o2)*, has higher lysine and tryptophan content and hard endosperm which is less susceptible to mechanical and biological damage. Three experiments were conducted to characterize the phenotypic and genotypic characteristics of high lysine maize. In the first experiment two separate diallels including 7 white and 9 yellow QPM inbreds were evaluated in five southern USA environments to estimate the general (GCA) and specific combining abilities (SCA) for grain yield and to identify potential heterotic relationships among them. QPM hybrids yielded less than commercial checks. GCA effects across environments were non-significant for grain yield but highly significant for secondary traits. Best yielding hybrids resulted from crosses among inbreds from different programs (CIMMYT, Mexico; University of Natal, South Africa and TAMU, USA). In the second experiment testcrosses between QPM inbreds and Tx804, were evaluated for agronomic performance, aflatoxin resistance and quality. QPM inbreds in testcrosses have similar flowering dates, plant height, ear height and test weights but lower grain yield than normal checks. Population 69 inbreds and their testcrosses were least susceptible to aflatoxin. Aflatoxin in testcrosses was positively correlated with endosperm texture (0.67) and kernel integrity (0.60) but negatively correlated with grain yield (-0.30) and silking date (-0.50). Tryptophan content was negatively correlated with endosperm modification. Amino acid levels of inbred lines were significantly correlated with those of hybrids, but with low predictive value. In the third experiment 92 high lysine maize inbreds with different origins [Stiff Stalk, Non Stiff Stalk, Pop 69, temperate (Tx802, Tx804, Tx806, B97, B104) and exotic subtropical lines (CML161, Do940y and Ko326y)]

were haplotyped on a cM scale utilizing 43 mapped SSR markers to characterize genetic diversity on chromosome 7, estimate linkage disequilibrium around *opaque-2* locus and determine the parental contribution in some inbreds. Dendrograms of genetic similarity showed clusters in agreement with the different origin of inbreds. A total of 200 alleles were detected with an average of 4.7 alleles/locus. Significant linkage disequilibrium was detected around *opaque-2* locus. Parental contributions of haplotypes showed segments of chromosome 7 exclusively contributed by one or the other parent.

DEDICATION

This dissertation is lovingly dedicated to my dear Father and dear Mother.

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Words cannot express alone my heartfelt thanks and sincere appreciation to Dr. Javier F. Betrán, chairman of my graduate committee for his constant guidance, support and encouragement all through the duration of my graduate study program. I appreciate his helping me pursue my doctoral studies in plant breeding at Texas A&M. I also deeply appreciate the kind financial assistance by Dr. Betrán through the maize breeding program to support my studies, especially in the last semester of my doctoral program, without which it would not have been possible for me to complete my studies.

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CHAPTER I

INTRODUCTION

Maize *(Zea mays* L.) is ranked at the top among the world cereal crops in production and consumption (USDA-FAS, 2003). United States is the largest producer of maize in the world producing about 10.3 billion bushels per year valued at 30 billion dollars (USDA-FAS, 2003). Maize is also the chief export crop of the U.S. Most of the maize produced in the United States (95%) is of the yellow dent type of which almost 58% is used primarily for livestock feeding. Other types of maize include popcorn, sweet maize, blue, white, high-oil, nutritionally dense, high-amylose and other types used in the production of food products such as maize bread and tortillas. Globally maize contributes 15% (representing more than 50 million tons) of the protein and 20% of the calories derived from food crops in the world's diet (National Research Council, 1988). In many developing countries in Latin America, Africa and Asia, maize is the staple food and sometimes the only source of protein in diet, especially in weaning food for babies. However, nutritional quality of maize protein is poor because of deficiencies of the essential amino acids lysine, tryptophan, and methionine (Glover and Mertz, 1987; Watson, 1988). Main reasons for poor quality of normal maize is the relatively high concentrations of prolamines or zeins storage proteins (50-60%) which are almost devoid of lysine and tryptophan causing maize to be nutritionally inferior in protein quality as compared with rice, wheat and other major cereals. The other storage proteins in the maize endosperm are albumins (3%), globulins (3%) and glutelins (30-45%) that have a relatively higher lysine content of 5-6%, 5-8% and 4-5%, respectively (Wilson, 1991). Lysine is the first limiting amino acid followed by tryptophan and threonine in the diets of non-ruminants and humans (Shimada & Cline, 1974). Lysine could also be limiting in poultry diet if protein sources other than soybean meal are used (Johnson et al., 2001). Increasing the levels of these nutritionally limiting amino acids is an

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This dissertation follows the style and format of Crop Science.

important objective of plant breeding programs. It has been estimated that substituting normal maize with high lysine maize on an equal weight basis for growing pigs and sows can diminish the use of synthetic lysine in animal feeds to maintain proper amino acid balance. In the United States, where maize is mainly used for feeding animals some kind of additive has to be used, mainly soybean meal to supplement the lysine and trytophan requirements of animal diet. Since quality protein maize (QPM) has a much superior protein quality (82.1 % as percent casein) it is postulated that the food processing and animal industries of the state of Texas alone could benefit by almost $80 million per year by replacing normal maize with QPM (TAES, 1990).

Discovery of the *opaque-2 gene* (Mertz et al., 1964) and subsequent efforts by the International Center of Maize and Wheat Improvement (CIMMYT), to develop maize inbreds having the *opaque-2* gene along with modifier genes that confer hard vitreous kernel texture and simultaneously selecting for superior agronomic characteristics have led to the development of QPM. Currently a wide variety of subtropical and tropical populations, pools and hybrids developed by CIMMYT are being used extensively in several countries including Brazil, China, India, Ghana and Central and South America, that have competitive yield and better quality. Texas A&M has an extensive QPM hybrid and inbred line development program. Currently several high lysine inbreds having different levels of endosperm hardness and adaptation to temperate Southern U.S. growing conditions have been developed. Main approaches used for development of QPM inbreds are selection within the CIMMYT germplasm, conversion of standard U.S. inbreds to QPM, and recycling of QPM inbreds. Major emphasis of the program is adaptation to temperate U.S. conditions, normal kernel phenotype, increased grain yield, and aflatoxin resistance.

Several breeding programs outside the USA are actively developing QPM inbreds, predominantly at CIMMYT, Mexico and University of Natal, South Africa. Some of CIMMYT's tropical and subtropical germplasm with intermediate and early maturity has desirable kernel quality characteristics and can significantly enhance the nutritional value of temperate maize germplasm for both food and feed purposes (Vasal,

2001). Furthermore, QPM hybrids have been reported to be less susceptible to aflatoxin, a potent carcinogen that causes losses worth millions of dollars in the southern USA, than current commercial hybrids (Bhatnagar et al., 2003). Despite the nutritional quality advantages and improved abiotic and biotic stress tolerance of exotic QPM, very little effort has been made to characterize and introgress exotic QPM germplasm into temperate U.S. maize germplasm. Characterization and selection for adaptation of these subtropical and tropical white and yellow QPM inbreds and a systematic introgression into temperate germplasm could enhance protein quality, increase genetic variability for quality, improve productivity, and be a source of valuable genes for abiotic and biotic stress resistance.

Preharvest aflatoxin contamination during flowering and grain filling period is a major problem for maize growers in Texas. Aflatoxin is a potent carcinogen produced by a fungus *Aspergillus flavus* and are a serious risk to human and animal health causing liver cancer (Castegnaro and McGregor, 1998). The maximum acceptable level of aflatoxin contamination of grain maize for food purposes is 20 ppb and for feed to animals is 300 ppb. There is immense variability in response and aflatoxin accumulation of maize hybrids, inbreds and cultivars. Commercial hybrids have been reported to show differences in aflatoxin accumulation but none are available for cultivation under conditions conducive for disease. Prediction of the response of a hybrid is complicated by many factors such as differences in environmental conditions, planting date, harvest date and insect injury. In general it has been found that hybrids more adapted to the region of growing with a good husk coverage and insect resistance accumulate less aflatoxin. Limiting factors in breeding for aflatoxin resistance are the spatial and temporal variation in aflatoxin accumulation that requires inoculation and a high number of replications, the lack of a reliable and inexpensive screening methodology, and the low metabolic activity of maize plants after physiological maturity (Payne, 1992). CIMMYT QPM inbreds and hybrids have been reported to be good candidates for introgression of aflatoxin resistance genes into temperate germplasm (Betran et al., 2002; Bhatnagar et al., 2003).

Understanding the genetic diversity of maize both at the phenotypic and genotypic level is crucial for plant breeders to develop efficient strategies for plant selection and introgression. Development of molecular markers has contributed extensively to the understanding of the genetic diversity of the maize genome and facilitated the study of the effects of past selection history, genetic drift, recombination, populations structures in maize germplasm, estimating genetic relationships between inbreds and the extent of haplotype sharing within diverse groups of maize inbreds when pedigree data is available. Inbreds in maize have also been a valuable resource in development of linkage maps and mapping quantitative trait loci. Currently efforts are on to map the maize genome at several laboratories and a great deal of information about mapped markers (SSR's RFLPs and SNPs) has been deposited at (http://www.maizegdb.org) which is publicly available.

This study includes three different experiments that are discussed in chapters II, III and IV. In the first experiment (Chapter I), two diallel experiments were conducted to evaluate 7 white and 9 yellow maize inbreds from different breeding programs (CIMMYT, Mexico; University of Natal, South Africa and TAMU, USA) in five southern USA environments to estimate the general (GCA) and specific combining abilities (SCA) for grain yield and secondary traits and to identify potential heterotic relationships among them. In the second experiment (Chapter II), testcrosses developed from crossing high lysine maize inbreds developed at Texas A&M with Tx804 were evaluated for agronomic performance, resistance to aflatoxin and protein quality. In the third experiment (Chapter III), 92 high lysine maize inbreds were characterized for haplotype variations along chromosome 7, particularly around the *opaque-2* locus, the level of genetic diversity of these inbreds in chromosome 7, extent of linkage disequilibrium around the *opaque-2* locus and along chromosome 7, and parental contribution in some inbreds.

CHAPTER II

COMBINING ABILITIES OF QUALITY PROTEIN MAIZE INBREDS*

OVERVIEW

Development and adoption of quality protein maize (*Zea mays* L.) (QPM) would increase the nutritional value of food and feed maize products. Breeding programs at CIMMYT, Texas A&M University (TAMU), and South Africa (SA) have developed high lysine inbreds. Information about how elite QPM inbreds of different origins combine and perform in hybrids will facilitate the selection of parents and breeding strategies for hybrid development. Our objectives were to estimate the general (GCA) and specific combining abilities (SCA) for grain yield and secondary traits among high lysine inbreds from different sources and to identify potential heterotic relationships among them. Seven white and nine yellow QPM inbreds were evaluated in two separate diallel experiments in five southern USA environments. QPM hybrids yielded less than commercial checks. GCA effects across environments were non-significant for grain yield but highly significant for agronomic and kernel quality traits. Based on GCA effects, TAMU inbreds had earlier maturities, shorter plants, and less grain moisture content than more subtropical CIMMYT and SA inbreds. The best yielding hybrids and highest SCA effects resulted from crosses among inbreds from different programs: TxX124 x CML 176, Tx811 x CML 181, Bo59w x CML 184 among the white hybrids, and Tx802 x Do940y among the yellow hybrids. QPM inbreds developed in different programs could represent potential heterotic groups for use in hybrid development and introgression of germplasm.

INTRODUCTION

Globally maize contributes 15% (representing more than 50 million tons) of the protein and 20% of the calories derived from food crops in the world's diet (National Research Council, 1988). In many developing countries in Latin America, Africa, and Asia, maize is the staple food and sometimes the only source of protein in diet, especially in weaning food for babies. Normal maize, being deficient in amino acids lysine and tryptophan that are essential for monogastric animals and humans, is nutritionally poor with a biological value (BV) of 40 - 57% (Bressani, 1992). High lysine maize with homozygous embryo and endosperm for mutant alleles o_2 at the α-zeins regulatory gene *opaque-2* shows about 60 to 100% increase in lysine and tryptophan and a higher BV (80% as compared with casein). Substituting normal maize with high lysine maize on an equal weight basis for growing pigs and sows can diminish the use of synthetic lysine in animal feeds to maintain proper amino acid balance (Asche et al., 1985; Burgoon et al., 1992; Knabe et al., 1992). In the USA, doubling lysine content in maize alone can add an estimated annual gross value of $360 million per year and can go up to $480 million per year if protein also is increased (Johnson et al., 2001).

There is an increasing number of elite exotic QPM inbreds being developed outside the USA. Therefore, characterization and selection for adaptation of these subtropical and tropical white and yellow QPM inbreds and a systematic introgression into temperate germplasm could enhance protein quality, increase genetic variability for quality, improve productivity, and be a source of valuable genes for abiotic and biotic stress resistance. Some of CIMMYT's tropical and subtropical germplasm with intermediate and early maturity has desirable kernel quality characteristics and can significantly enhance the nutritional value of temperate maize germplasm for both food and feed purposes (Vasal, 2001). Furthermore, QPM hybrids have been reported to be less susceptible to aflatoxin, a potent carcinogen that causes losses worth millions of dollars in the southern USA, than current commercial hybrids (Bhatnagar et al., 2003). Introgression of exotic germplasm into temperate adapted maize has been widely

emphasized as a method to expand genetic diversity of maize germplasm in the USA (Goodman et al., 2000). Despite the nutritional quality advantages and improved abiotic and biotic stress tolerance of exotic QPM, very little effort has been made to characterize and introgress exotic QPM germplasm into temperate U.S. maize germplasm. Major reasons for under-utilization of exotic germplasm, particularly QPM germplasm, are photoperiod sensitivity, poor standability, and low grain yield in comparison with temperate adapted germplasm (Bhatnagar et al., 2003). Before incorporating exotic QPM germplasm into temperate areas, an initial evaluation of exotic germplasm is useful to determine their breeding potential (Geadelmann, 1984). Diallels between elite exotic and temperate QPM inbreds can help in introgression of useful quality traits from tropical high lysine maize into temperate germplasm will be to identify suitable parental inbreds that would combine well to make superior hybrids. Successful exploitation of heterosis, usually expressed as the superiority of F_1 over some measure of performance of its parents, has been regarded as the primary factor for the success of modern commercial maize industry in the U.S. (Stuber, 1994b). Differences between the parents and their crosses are quantified as mid-parent or high-parent heterosis. Identifying heterotic groups and characterizing heterotic patterns among parents is perhaps one of the most costly and tedious activity in a maize breeding program. A heterotic group has been defined as "a collection of germplasm that, when crossed with germplasm from an external group, tends to exhibit a higher degree of heterosis (on average) than when crossed with a member of its own group" (Lee, 1995).

Information on agronomic performance, combining abilities, and heterotic relationships of elite subtropical and tropical QPM parents developed at CIMMYT and in South Africa with temperate inbreds adapted to southern USA environments will facilitate their incorporation and introgression. Therefore, our objectives were to: (1) estimate GCA effects for grain yield and agronomic traits of QPM inbreds originated in subtropical (CIMMYT, Mexico and University of Natal, South Africa) and temperate (TAMU) breeding programs and (2) estimate SCA effects, and identify best hybrid combinations and possible heterotic relationships among these inbreds.

REVIEW OF LITERATURE

Several authors have reviewed the dominant heterotic patterns prevalent in major maize production regions of the world (Wellhausen, 1978; Ron Parra and Hallauer, 1997). Important and well-characterized heterotic patterns established over a period of extensive breeding history include the Reid Yellow Dent x Lancaster Sure Crop in the U.S. and European flint x U.S. dent in Europe. A step further in characterizing and classifying most of temperate maize in the U.S. based on varying regional breeding histories and heterotic patterns has given rise to several families and lineages (B73, Mo17, Oh43, C103) within the heterotic groups. In comparison, heterotic patterns in tropical maize are more diverse and only recently characterized between Tuxpeño and several other populations such as ETO composite, Suwan 1, Cateto, Coastal tropical flints, and Cuban flints (Wellhausen, 1978; Goodman, 1985; Hallauer et al., 1988) can help determine heterotic relationships among exotic and temperate QPM inbreds, which are at present relatively unknown, and identify the best hybrids for both production and breeding purposes. In the USA, more than 20 years ago after the discovery of *o2* mutant effects, breeding programs converted normal inbreds and populations to their opaque-2 soft counterparts (NTR1, NTR2, BSAA-*o2*, B73o2, SSSS-*o2*) (Mertz et al., 1964). After this initial effort, the interest in QPM or high lysine maize decreased and it has since remained low. To our knowledge, only Crow's Hybrid Seed Company has continuously conducted a breeding program to improve high lysine maize. Texas A&M University has also maintained a breeding program to develop QPM inbreds and hybrids with normal seed appearance, competitive yield, and adaptation to southern USA (Betrán et al., 2003c, 2003d, 2003e).

The International Center for Maize and Wheat Improvement (CIMMYT) has developed QPM that has improved kernel quality characteristics over *o2/o2* soft genotypes, by introducing modifier genes and selecting for a hard, vitreous endosperm in *o2/o2* germplasm (Vasal, 2001). CIMMYT QPM populations, pools, inbreds, and hybrids adapted to subtropical and tropical environments are widely used in the

development of high lysine maize in Brazil, China, Ghana, India, and several Latin American countries (Vasal, 2001). The maize breeding program at the University of Natal, South Africa has developed high lysine white (e.g., Bo46W and Bo59W) and yellow inbreds (e.g., Do940y and Ho4664) that produce hybrids competitive in yield with normal hybrids and tolerant to diseases (Gevers and Lake, 1992).

QPM germplasm, which are mostly tropical and subtropical in adaptation, are less adapted to temperate areas. Previous studies have shown that there is a significant yield gap between subtropical/tropical QPM and temperate adapted QPM hybrids, however white QPM hybrids in general are more competitive for yield in subtropical environments as compared to temperate environments (Bhatnagar et al., 2003). With increasing latitude and day length QPM hybrids tend to have more biomass, higher ear placements and higher grain moisture content at maturity. Vasal (1993) in a 10-parent diallel study of tropical white QPM germplasm conducted in three environments in Mexico and USA reported similar results. Studies on combining ability of CIMMYT QPM lowland tropical and subtropical germplasm conducted in several environments (Mexico, Guatemala, Colombia, Philippines and U.S.) showed significant GCA effects for grain yield, plant height, days to silk and endosperm hardness (Vasal et al., 1993). SCA effects were non-significant for grain yield and endosperm hardness suggesting additive gene action for these traits. Similar results were reported by Beck et al. (1991). In contrast San Vincente et al. (1998) reported greater relative importance of non-additive genetic effects than additive genetic effects (62% vs. 38%) for grain yield in tropical white populations. In a 10 parent diallel of five tropical late and 5 subtropical intermediate white endosperm QPM evaluated at eight locations significant GCA effects were observed for grain yield, endosperm hardness and tolerance to ear rot (Tolessa et al., 1999). Hybrids involving QPM lines CML 176, CML 142 and CML 186 showed high grain yields and GCA effects. High SCA effects were observed between tropical x subtropical hybrids for grain yield and endosperm hardness. Studies on combining ability of tropical QPM inbreds derived from five pools and populations (Pools 23Q, 24Q, Pop.62, 63 and 64) showed on an average 14% higher grain yield and 60% more

tryptophan concentration in protein (Pixley and Bjarnason, 1993). Significant GCA effects were observed for grain yield, protein concentration in grain and tryptophan concentration in protein.

MATERIALS AND METHODS

Germplasm and environments

Two separate diallel experiments for white and yellow QPM inbreds developed in three QPM breeding programs, viz., CIMMYT, University of Natal, and TAMU, were evaluated in the 1999 growing season (February to September) in five southern USA environments. Diallel crosses among the lines were made in 1998 summer at College Station, TX and 1999 winter at Homestead, FL. Seeds from reciprocal crosses of the full diallel were bulked to form one set of hybrids because sufficient seed was not obtained for all the crosses. Twenty-one F_1 crosses (Griffing's method 4) among seven white QPM inbreds (Table 2.1), two commercial checks (Pioneer Brand P32H39 and Asgrow RX901W), and five experimental checks were evaluated at College Station, Weslaco, Castroville, Halfway, and Dumas, TX. Thirty-six F_1 crosses (Griffing's method 4) among nine yellow QPM inbreds (Table 2.1), four commercial checks, including Pioneer Brand hybrids P3223, P3394, and P32Y65 and DeKalb hybrid DK668, and eight experimental checks were evaluated at College Station, Corpus Christi, Granger, Wharton, and Dumas, TX. The characteristics of the environments and mean grain yield for both white and yellow diallels are described in Table 2.2. Standard cultural and agronomic practices generally used at all locations were applied.

Field measurements

Data were recorded on a plot basis for both white and yellow QPM diallel experiments on the following agronomic traits: grain yield (combine harvested grain weight expressed in Mg ha^{-1} and standardized to 155 g kg^{-1} moisture content), silking date (number of days from planting until 50% of the plants showing silks), plant height

(distance in cm from the ground to the top of tassel), ear height (distance in cm from the ground level to the main ear-bearing node), root lodging (% plants leaning at an angle greater than 30% from the vertical), stalk lodging (% plants with broken stalks at or below the main ear at maturity), grain moisture (g kg^{-1} moisture of grain at harvest), and test weight (recorded as g pint^{-1} by standard equipment and converted to kg m^{-3}).

Table 2.1. White and yellow maize inbreds involved in two diallel experiments evaluated in five southern USA environments during 1999.

Parental line	Source	Adaptation
White Inbreds		
CML 176	CIMMYT	Subtropical
CML 181	CIMMYT	Subtropical
CML 184	CIMMYT	Subtropical
TxX 124	Texas A&M	Southern USA
Tx807	Texas A&M	Southern USA
Tx811	Texas A&M	Southern USA
Bo59w	South Africa	Subtropical
Yellow Inbreds		
CML 190	CIMMYT	Subtropical
CML 193	CIMMYT	Subtropical
Tx802	Texas A&M	Southern USA
Tx814	Texas A&M	Southern USA
Tx818	Texas A&M	Southern USA
Tx820	Texas A&M	Southern USA
Do940y	South Africa	Subtropical
TxX 808	Texas A&M	Southern USA
TxX 810	Texas A&M	Southern USA

For white QPM hybrids, ear samples from competitive plants in a single replication per environment were collected at harvest time and used to measure the following kernel quality traits (Serna-Saldivar et al., 1991): 1000-kernel weight (in g), endosperm hardness (recorded as % of kernel weight removed using a tangential abrasive dehulling device (TADD) to remove the pericarp uniformly using 45 g samples

Table 2.2. Characteristics and mean grain yield for environments used to evaluate white and yellow QPM diallel hybrids and non-QPM checks in southern USA in 1999.

Environments	Code	Type of diallel evaluated	Latitude	Longitude	Elevation	Plot area	Water regime	Grain yield White diallel	Grain yield Yellow diallel
					m	m^2/plot		Mg ha^{-1}	Mg ha^{-1}
WESLACO, TX	WE	White	26°09'N	97°59'W	22.5	11.85	Irrigated	6.05 $\pm$ 0.05	-
CORPUS CHRISTI, TX	CC	Yellow	27°48'N	97°23'W	12.9	13.25	Rainfed	-	3.82 $\pm$ 0.09
WHARTON, TX	WH	Yellow	29°17'N	96°13'W	30.3	15.73	Rainfed	-	6.03 $\pm$ 0.06
CASTROVILLE, TX	CA	White	29°21'N	98°52'W	228.2	14.26	Irrigated	4.98 $\pm$ 0.09	-
COLLEGE STATION, TX	CS	White & Yellow	30°37'N	96°20'W	96.0	9.95	Irrigated	4.92 $\pm$ 0.18	5.96 $\pm$ 0.09
GRANGER, TX	GR	Yellow	30°43'N	97°26'W	172.4	15.00	Rainfed	-	6.99 $\pm$ 0.05
HALFWAY, TX	HA	White	34°11'N	101°57'W	1071.0	9.60	Irrigated	3.70 $\pm$ 0.26	-
DUMAS, TX	DU	White & Yellow	35°51'N	101°58'W	1098.2	11.74	Rainfed	8.48 $\pm$ 0.26	9.42 $\pm$ 0.20

of whole kernels and dehulling in the TADD for 10 minutes); floaters (recorded as % kernels floating in a 1.275 g/cc sodium nitrate solution); pericarp removal (performed by cooking 25 g sample in a steam kettle containing 1% lime (calcium hydroxide) for 20 minutes at boiling point, washing the samples and staining them with eosine and methyl blue solutions to differentiate between pericarp (blue-green) and endosperm (light pink). The samples were later rated on a scale of 1-5 (1 = complete removal and 5 = 100% pericarp retained) for the extent of pericarp removal. Endosperm hardness is related to the proportion of hard endosperm to soft endosperm and it is an important quality trait for the milling industry. Percent floaters and pericarp removal are related to endosperm hardness and cooking time for production of masa used in making tortilla and tortilla chips (Serna-Saldivar et al., 2001).

Statistical analyses

Both white and yellow diallel experiments were planted in 2-row plots following an alpha lattice experimental design with two replications per environment. Individual analyses of variance per environment and across environments were conducted using PROC GLM (SAS Institute Inc., 1997). Hybrids were considered fixed effects, and environments and replications random effects. Significance of hybrid, GCA, and SCA mean squares were estimated with *F* tests using their interaction with the environment as error term. General combining ability effects of the parents and SCA effects for the crosses as well as their mean squares at each environment and across environments were estimated following Griffing's method 4 diallel analysis (Griffing, 1956) using a computer program originally written by Dr. S.G. Carmer (University of Illinois) and later modified and adapted by Dr. Hector Barreto at CIMMYT.

Biplots were constructed for both white and yellow diallel crosses using mean grain yield across locations to visualize relationships among parental inbreds in hybrid combinations and identify possible heterotic associations among them. Since parental inbred *per se* were not included in the diallel analysis, mean grain yield of inbreds in hybrids was used as inbreds values for calculations. Biplots are commonly used to

analyze two-way data where rows and columns represent different experimental units (e.g., genotypes and environments, inbred A x Inbred B, etc.). In a diallel-cross data, both columns and rows represent the same parental inbreds, which are both an entry and a tester. Principal component scores (PC1 and PC2) were derived using PROC PRINCOMP (SAS Institute Inc., 1997), following methods described by Yan and Hunt (2002), and used to construct the biplot (Appendix A). A polygon was drawn connecting entries located furthest from the origin in each biplot. Subsequently, this polygon was divided into sectors by perpendiculars (A, B, C, and D) drawn from the origin to each side of the polygon. All testers and entries included in the same sector represent good hybrid combinations and potential heterotic groups for grain yield. The best hybrid in any sector is defined by the vertex entry and the tester that is located furthest from the origin. SCA effects between entries and testers in any sector can be visualized by projecting an entry onto the vector of the tester or its extension.

RESULTS AND DISCUSSION

White hybrids

Significant differences among hybrids across environments were observed for all traits (Table 2.3). Mean grain yield across environments was 5.62 Mg ha^{-1} for the hybrids, 5.70 Mg ha^{-1} for QPM hybrids, and 6.27 Mg ha^{-1} for non-QPM checks. Significant differences among the QPM crosses and non-QPM checks were observed for days to flowering, plant and ear height, and grain moisture. QPM crosses, on average, flowered five days later (80.66 vs. 75.55 days), were taller (235.27 cm vs. 220.0 cm), had higher ear placement (98.92 cm vs. 80.38 cm), and higher grain moisture content (185.74 vs. 156.59 g kg^{-1}) than non-QPM checks.

Significant differences among diallel hybrids were observed for all traits, except grain moisture (Table 2.3). Significant differences among GCA effects were observed for all agronomic traits except grain yield, whereas SCA effects were significant for grain yield and stalk lodging. Hybrids x environment effects were significant for all

Table 2.3. A combined analysis of variance and means for grain yield and agronomic traits of white hybrids across five southern USA environments.

Source of variation	df	Mean squares						
		GY†	SD	PH	EH	RL	SL	GM
		Mg ha^{-1}	d	cm	cm	%	%	g kg^{-1}
Environment	4	181.34**	5190.56**	125417.22**	55861.62**	5682.99**	7255.39**	289917.29**
Reps/Env	5	4.25	0.41	259.43	118.91	206.78	140.66	1217.41
Hybrids	27	4.30*	27.35**	1746.77**	966.58**	383.64*	200.69**	2506.21**
F$_1$ diallel hybrids	20	3.24*	11.54**	1652.38**	812.00**	387.86*	177.34**	2152.14
GCA	6	1.18	31.72**	4810.64**	2373.22**	935.32*	281.97**	4983.92*
SCA	14	3.98**	2.90	304.76	141.23	152.66	131.98**	965.64
Checks	6	7.67	74.38**	1898.71**	1104.13**	421.99	306.91**	3906.18*
F$_1$ diallel hybrids vs. checks	1	5.33	61.34**	2722.70**	3233.03**	69.11	30.44	1187.74
Hybrids*Env	108	2.46**	2.91**	205.42**	145.94**	215.70**	69.54**	1209.47**
F$_1$ diallel hybrids x Env	80	1.76	3.30**	183.61**	114.13**	204.31**	66.49*	1278.46**
GCA x Env	24	2.18*	5.16**	203.68**	106.06**	393.99**	64.08	1806.75**
SCA x Env	56	1.61	2.51**	173.55**	118.00**	122.96	67.66*	1043.01**
Checks x Env	24	4.75**	1.96*	213.81**	194.06**	282.49**	68.56*	1067.02**
F$_1$ diallel hybrids vs. Checks x Env	4	1.77	0.57	393.87**	328.64**	868.92**	90.97	342.15
Error	135	1.35	1.16	65.52	54.37	81.58	42.58	388.07
Mean for all hybrids		5.62	80.39	236.28	98.16	7.19	10.79	184.42
Mean for QPM hybrids		5.70	80.66	235.27	98.92	6.87	10.60	185.74
Mean for non-QPM hybrids		6.27	75.55	220.00	80.38	3.19	5.93	156.59
LSD (0.05)		1.03	0.95	5.03	6.52	8.95	5.50	20.53
CV (%)		9.23	0.60	1.07	3.36	62.85	25.71	5.61

*,** Significant at $P < 0.05$ and 0.01, respectively.
† GY, grain yield; SD, silking date; PH, plant height; EH, ear height; RL, root lodging; SL, stalk lodging; GM, grain moisture.

Table 2.4. A combined analysis of variance and means for kernel traits of white hybrids across five southern USA environments.

Source of variation	df	Mean squares				
		TW†	TKW	HD	F	PR
		kg m^{-3}	g	%	%	scale 1-5
Environment	4	13632.74**	16430.07**	286.95**	5742.65**	4.26**
Hybrids	27	1279.61**	5419.77**	37.41**	985.68**	1.53**
F$_1$ diallel hybrids	20	1280.24**	4278.03**	35.07**	1075.48**	1.18**
GCA	6	2921.32**	12466.18**	95.84**	2654.61**	3.46**
SCA	14	575.97**	770.21	9.12	398.72	0.21
Checks	6	1437.95**	8976.68**	33.43**	705.36**	2.56**
F$_1$ diallel hybrids vs. checks	1	316.68	6913.37**	108.05**	871.49**	2.29*
Hybrids*Env‡	108	208.69	618.10	5.17	213.11	0.25
Means for all hybrids		754.31	284.04	40.01	44.84	3.88
Means QPM crosses		753.02	281.84	40.09	45.34	3.85
Means non-QPM checks		772.33	312.61	38.87	38.40	4.22
LSD (0.05)		18.11	31.17	2.85	18.30	0.63
CV (%)		1.92	8.75	5.68	32.56	12.89

*,** Significant at *P* < 0.05 and 0.01, respectively.

† TW, test weight; TKW, 1000-kernel weight; HD, endosperm hardness; F, floaters; PR, pericarp removal (1 = complete removal and 5 = 100% pericarp retained)**.**

‡ Hybrids*Env was used as the error term to estimate the significances of all the *F* tests.

Table 2.5. General combining ability effects of seven white inbreds for grain yield (per environment and across environments), agronomic and kernel traits across five southern USA environments.

Inbreds	Grain yield					
	CS†	WE	CA	HA	DU	Across
	----------------------Mg ha^{-1}---------------------					
CML 176	0.44	-0.09	0.32	-0.23	0.63	0.21
CML 181	-0.17	-0.60**	-0.84*	-0.05	0.75	-0.19
CML 184	-0.09	-0.19	0.41	0.01	-0.03	0.01
TxX124	0.22	0.11	0.47	0.07	0.15	0.19
Tx807	-0.23	0.54**	0.43	-0.17	-1.01	-0.09
Tx811	-0.51	0.47**	-0.08	0.07	-0.03	-0.03
Bo59w	0.34	-0.24	-0.70*	0.29	-0.45	-0.13

† CS, College Station; WE, Weslaco; CA, Castroville; DU, Dumas; HA Halfway.

‡ SD, silking date; PH, plant height; EH, ear height; RL, root lodging; SL, stalk lodging; GM, grain moisture; TW, test weight; TKW, 1000-kernel weight; HD, endosperm hardness; F, floaters; PR, pericarp removal (1 = complete removal and 5 = 100% pericarp retained).

Table 2.5. Continued

SD‡	PH	EH	RL	SL	GM	TW	TKW	HD	F	PR
d	cm	cm	%	%	g kg^{-1}	kg m^{-3}	g	%	%	scale 1-5
0.85*	7.05**	2.06	8.08*	-0.89	20.1*	17.92**	-17.91*	0.16	-6.88	0.49**
0.85*	1.13	6.35**	-4.46	0.37	-16.7*	-10.68**	22.85**	1.84**	15.16**	-0.07
-0.95*	-7.22**	-10.24**	0.90	-2.64**	1.8	5.14	9.53	-0.40	-1.20	0.27*
0.05	9.16**	8.73**	-5.08	-1.37	-0.1	8.04*	20.01*	-3.98**	-13.00**	-0.25*
-0.17	-17.15**	-5.88**	-3.00	-1.64*	-0.5	-1.98	-36.93**	-0.28	-6.52	-0.63**
-1.09*	-2.40	-4.03*	-0.78	1.98*	-6.2	-9.76*	-10.83	1.44*	12.12**	-0.01
0.49	9.43**	3.03	4.35	4.19**	2.5	-8.70*	13.17	1.20*	0.32	0.23

agronomic traits. GCA x environment effect was significant ($P < 0.05$) for grain yield and highly significant for all other agronomic traits ($P < 0.01$) except stalk lodging. SCA x environment effects were significant for silking date, plant height, ear height, and grain moisture. For quality traits, all GCA effects were significant whereas SCA effects were non-significant for all traits except test weight (Table 2.4).

GCA effects for grain yield showed significant variation between and within the three different groups of parental lines (CIMMYT, TAMU and SA), in different environments (Table 2.5). Weslaco and Castroville were the only two environments with significant GCA effects. The highest GCA effect for grain yield across environments was observed for CML176 (0.21 Mg ha^{-1}), but this was not significant. CML176 hybrids have shown high yield potential and low aflatoxin accumulation in Texas environments in previous studies (Betrán et al., 2002; Bhatnagar et al., 2003) and performed consistently well in trials conducted by CIMMYT across 29 locations in Latin America, Asia, and Africa (CIMMYT, 1999). CML176 and CML181 produced tall and late hybrids. CML176 has also an undesirable positive GCA effect for root lodging and an off-white grain color (data not shown). CML184 had negative GCA effects for days to flowering, and plant and ear height, which indicates that it is more suited for temperate environments than CML176 and CML181. Inbreds Tx807 and Tx811 had significant negative GCA effects for days to flowering, plant and ear height across environments indicating early maturity, shorter plants and lower ear placements than exotic QPM lines. Inbred Bo59w had significant GCA effects for stalk lodging and plant height.

Inbred CML176 had the most desirable significant GCA effect for test weight, CML181 for 1000-kernel weight, TxX124 for endosperm hardness and floaters, and Tx807 for pericarp removal (Table 2.5). Inbred TxX124 had the best combination of GCA effects for quality traits showing high GCA effects for test weight and 1000-kernel weight together with low GCA effects for endosperm hardness, floaters, and pericarp removal.

The top five performing crosses having high positive significant SCA effects and high grain yields were Tx811 X CML181 (SCA = 1.01**, 6.53 Mg ha^{-1}), Tx807 X

Fig. 2.1. Biplot for grain yield and putative heterotic relationships between seven white parental inbreds in hybrid combinations (entries◆ and testers■) across five southern USA environments.

CML181 (0.87**, 6.30 Mg ha^{-1}), Bo59W X CML184 (0.71**, 6.28 Mg ha^{-1}), TxX124 X CML176 (0.69*, 6.82 Mg ha^{-1}), and Bo59W X CML176 (0.41*, 6.18 Mg ha^{-1}). The first two principal component axes in the biplot for mean grain yield of seven inbreds in entry x tester hybrids across environments explained 42.6 % and 35.6 % of the total variation, respectively (Fig. 2.1). Entries Tx811, CML176, Bo59W, and CML181, which are

located furthest from the origin, defined a polygon that was divided into four sectors by perpendiculars A, B, C, and D. In sector AB, the best hybrid combination was the vertex entry Tx811 x tester CML181.   Another good hybrid in sector AB was Tx807 x CML181. In sector BC, the best hybrid was the vertex entry CML176 x Bo59W. Other good hybrids in this sector were CML176 x TxX124 and CML184 x Bo59W. Sectors CD and DA showed similar responses as observed for sectors AB and BC, respectively. Potential heterotic groups for southern USA could involve crosses between Texas inbreds (Tx811 and Tx807) and subtropical and tropical QPM inbreds (CML181, CML176, and CML184), and between CIMMYT lines CML176 and CML184, and SA inbred Bo59W.

Yellow hybrids

Significant differences among hybrids across environments were observed for all traits except root lodging (Table 2.6). Mean grain yield across environments was 6.44 Mg ha^{-1} for all hybrids and 6.25 Mg ha^{-1} for QPM hybrids, which was significantly lower than that for non-QPM crosses (8.18 Mg ha^{-1}). QPM hybrids across environments flowered approximately 3 days later (81.41 days vs. 78.55 days) and had higher grain moisture content (169.61 g kg^{-1} vs. 143.89 g kg^{-1}) than non-QPM hybrids. Vasal et al. (1993) found similar responses of subtropical yellow QPM populations for days to flowering and ear height in USA environments.

GCA effects were significant for all traits except grain yield and root lodging. Highly significant differences for SCA effects were observed for grain yield, plant height, and test weight. GCA x environment effects were highly significant for all traits, whereas SCA x environment effects were significant for all traits, except days to flowering, root lodging, and test weight.

GCA effects for all yellow QPM inbreds varied significantly across environments (Table 2.7). Inbreds CML190 and CML193 had negative GCA effects for grain yield in most environments except CML190 at Granger, indicating their lack of adaptation to USA environments. Texas inbreds had positive GCA effects in most

Table 2.6. A combined analysis of variance and means for grain yield and agronomic traits of yellow hybrids across five southern USA environments.

Source of variation	df	Mean squares							
		GY†	SD	PH	EH	RL	SL	GM	TW
		Mg ha^{-1}	d	cm	cm	%	%	g kg^{-1}	kg m^{-3}
Environment	4	327.28**	4986.93**	155869.58**	74939.46**	24007.74**	1275.35**	169403.90**	156000.27**
Reps/Env	5	1.15	1.16	520.96	122.42	202.49	150.98	2011.56	1560.23
Hybrids	39	7.35**	26.41**	742.32**	270.38**	164.74	119.44*	2368.98**	2136.63**
F$_1$ diallel hybrids	35	3.38**	18.35**	771.61**	238.51**	156.12	122.09*	1943.58**	2176.38**
GCA	8	4.46	67.76**	2172.83**	519.93*	468.14	325.90*	6718.57**	4750.27*
SCA	27	3.10**	3.72	356.39**	155.16*	64.34	61.61	499.96	1977.62**
Checks	3	11.87**	30.96**	396.67	253.97	108.20	11.52*	96.13	311.38
F$_1$ diallel hybrids vs. checks	1	132.55**	294.69**	754.84	1434.24	636.38	350.31	24076.69*	6250.88
Hybrids x Env	156	1.74**	5.21**	279.74**	127.61**	145.98**	71.45**	731.25**	1068.31**
F$_1$ diallel hybrids x Env	140	1.70**	5.18**	236.26**	118.90**	135.65**	77.87**	723.91**	1086.53**
GCA x Env	32	2.41**	11.74**	499.61**	227.99**	386.60**	143.52**	1602.95**	1782.18**
SCA x Env	108	1.48*	3.25	158.19**	86.51*	61.13	58.45**	470.65**	740.37
Checks x Env	12	0.75	3.40	168.79	74.11	152.52**	2.73	75.55	642.64
F$_1$ diallel hybrids vs. Checks x Env	4	6.14**	11.62**	2134.44**	594.64**	487.87**	52.82	2955.54**	1707.65*
Error	195	1.08	2.03	100.90	66.31	62.03	34.38	259.08	703.93
Mean for all hybrids		6.44	81.13	227.19	88.26	8.71	4.45	167.04	746.59
Mean for QPM hybrids		6.25	81.41	227.00	88.90	9.13	4.76	169.61	745.30
Mean for non-QPM hybrids		8.18	78.55	228.93	82.59	4.93	1.64	143.89	758.04
LSD (0.05)		1.15	1.26	8.19	6.37	5.17	4.09	12.22	24.07
CV (%)		5.24	0.50	2.98	4.63	78.81	96.46	2.29	1.55

† GY, grain yield; SD, silking date; PH, plant height; EH, ear height; RL, root lodging; SL, stalk lodging; GM, grain moisture; and TW, test weight.

*,** Significant at $P < 0.05$ and 0.01, respectively.

Table 2.7. General combining ability effects of nine yellow inbreds for grain yield (per environment and across) and agronomic traits across five southern USA environments.

Inbreds	Grain yield						SD‡	PH	EH	RL	SL	GM	TW
	CS†	CC	GR	WH	DU	Across	d	cm	cm	%	%	g kg⁻¹	kg m⁻³
	------------------------- Mg ha⁻¹ -------------------------						d	cm	cm	%	%	g kg⁻¹	kg m⁻³
CML 190	-0.41	-0.01	0.61*	-0.67**	-0.27	-0.15	-0.43	10.31**	2.24	1.91	1.80	2.33	21.24**
CML 193	-0.38	-0.41	0.54*	-0.33	-0.27	-0.38	1.19*	-1.78	-0.89	4.34	-0.30	5.93	10.81
Tx802	0.40	0.51*	0.51*	0.17	-0.02	0.31	-0.50	-4.12	-0.83	-0.90	-1.41	6.84	-12.23*
Tx814	0.28	0.38	0.07	0.43*	-0.22	0.19	-0.40	-1.01	-1.65	-0.29	-2.83	10.53*	-11.71*
Tx818	-0.22	-0.85**	-0.21	0.47*	-0.87	-0.34	-0.37	1.41	2.51	-3.35	3.22*	-6.41	2.70
Tx820	0.37	0.12	0.66*	-0.14	0.21	0.24	-0.70	5.25	3.67	-3.11	0.78	6.10	1.29
Do940y	0.34	0.41	-0.49*	0.16	-0.17	0.05	1.96**	2.01	1.98	2.72	-1.69	-1.13	11.71*
TxX 808	-0.26	-0.04	-0.07	0.36*	0.66	0.13	-1.08*	-8.71**	-4.46*	-1.28	2.46	-22.21**	-13.13*
TxX 810	-0.12	-0.12	-0.54*	-0.44*	0.95	-0.06	0.33	-3.36	-2.58	-0.04	-2.03	-1.98	-10.41

† CS, College Station; CC, Corpus Christi; GR, Granger; WH, Wharton; and DU, Dumas.

‡ SD, silking date; PH, plant height; EH, ear height; RL, root lodging; SL, stalk lodging; GM, grain moisture; and TW, test weight.
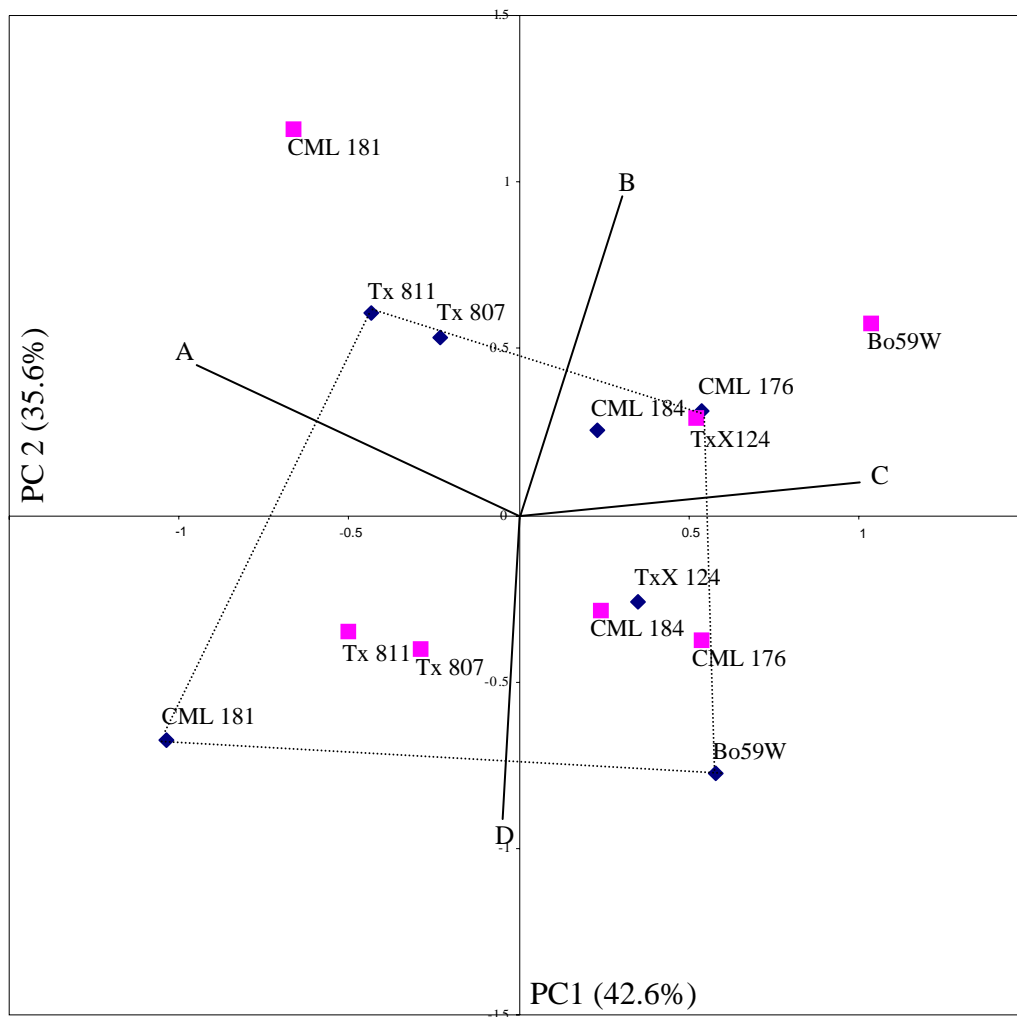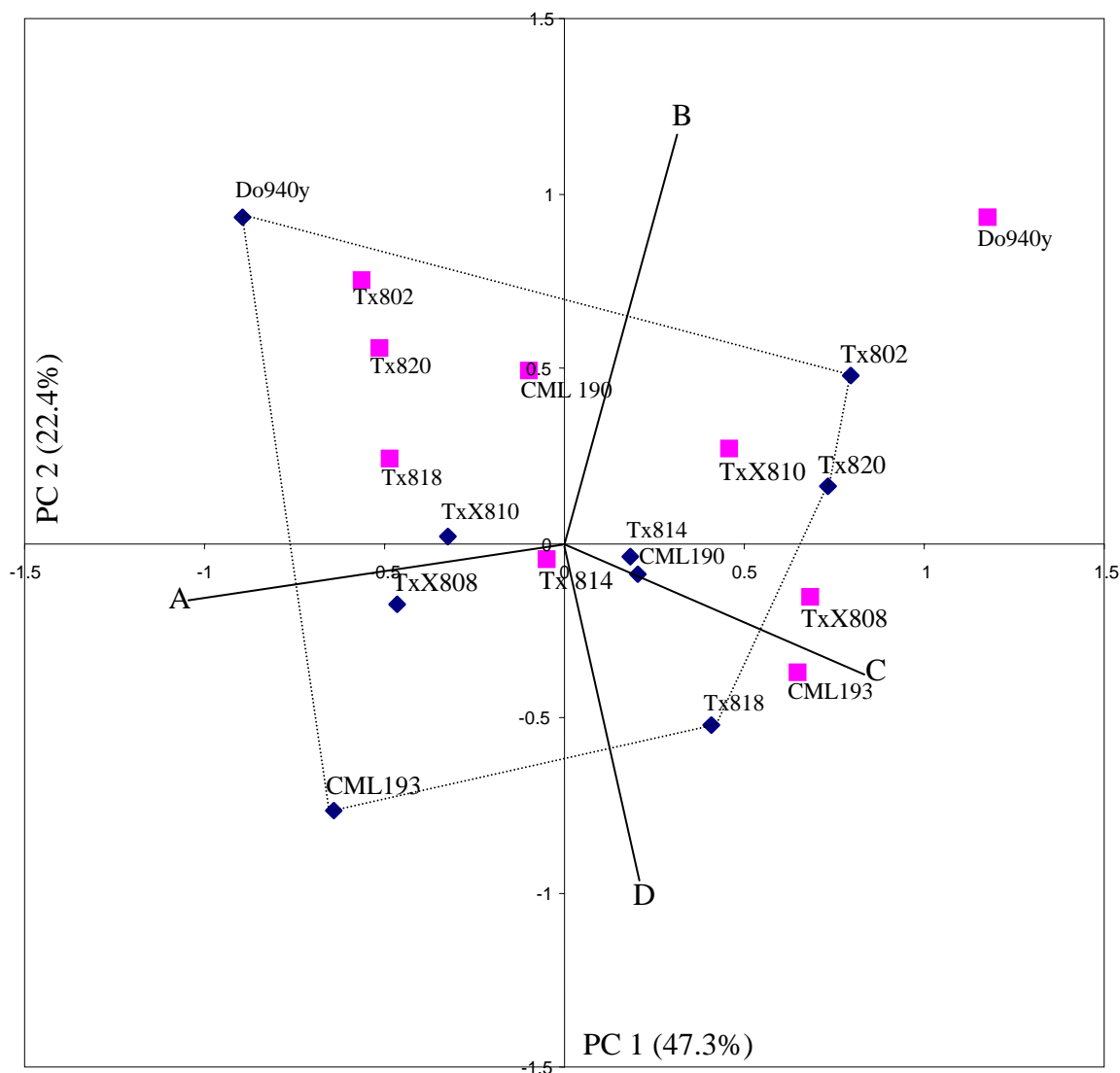
Fig. 2.2. Biplot for grain yield and putative heterotic relationships between nine yellow parental inbreds in hybrid combinations (entries ◆ and testers ■) across five southern USA environments.

environments except Tx818, which showed significant negative GCA effects at Corpus Christi and Dumas. In general, Tx802, Tx820, and Tx814 showed high GCA effects for grain yield. Tx802 showed consistently high positive GCA effects for grain yield at most environments.

CIMMYT inbreds showed variable GCA effects for agronomic traits across environments (Table 2.7). CML190 and Do940y had significant positive GCA effects for test weight and represent potential source germplasm to increase the test weight of Texas inbreds, such as Tx802 and Tx814 that had negative GCA effects for this trait. Texas inbreds, in general, had significant negative GCA effects for days to flowering (TxX808 and Tx820), plant height (Tx802 and TxX808), root lodging (Tx818 and Tx820), stalk lodging (Tx814), and grain moisture (TxX808). Do940y, a late maturing inbred, had significant positive GCA effect for days to flowering. The top three hybrid combinations having high positive SCA effects and high grain yields involved crosses between South African and Texas inbreds [Do940y X Tx802 (0.88**, 7.51 Mg ha^{-1}), Do940y X Tx818 (0.75**, 6.69 Mg ha^{-1}), and Do940y X Tx820 (0.66**, 7.24 Mg ha^{-1})]. The first two principal component axes in the biplot for mean grain yield for the nine yellow inbreds in entry x tester hybrids across environments explained 47.3 % and 22.4 % of the total variation, respectively (Fig. 2.2). Inbreds Do940y, Tx802, Tx820, Tx818, and CML193 defined a polygon that was divided into four sectors by the perpendiculars A, B, C, and D drawn to the sides of the polygon. In sector AB, the vertex entry Do940y showed a high positive response in hybrids with testers Tx802, Tx820, Tx818, and CML190. Sector BC showed similar relationships. The vertex entry CML193 in sector DA did not show any significantly high positive response with tester Tx814 that was located very close to the origin of the biplot. Inbred TxX808 in sector DA and TxX810 in sector AB, both derived from crosses between TAMU and SA inbreds, showed variable response with Texas lines. Two potential heterotic groups were identified in the biplot for yellow inbreds. The first group included Texas inbreds Tx802, Tx820, and Tx818, which combined well with SA inbred Do940y, and the second group included Do940y and CML190.

Successful breeding approaches are a direct consequence of the gene action prevalent in the breeding population under consideration. The relative importance of additive vs. non-additive effects for grain yield in diallel crosses is an indication of the type of gene action (Baker, 1978). In the two diallels reported here, the GCA effects for

grain yield across environments were not significant whereas SCA effects were highly significant (Table 2.3 and 2.6). An opposite trend was observed for quality and agronomic traits where GCA effects across environments were more important than SCA effects (Table 2.4). The type of more prevalent gene action for grain yield has been variable depending on the parents and environments under consideration in other studies. San Vincente et al. (1998) reported greater relative importance of non-additive genetic effects than additive genetic effects for grain yield in diallel crosses among improved tropical white endosperm populations. In contrast, additive genetic effects were prevalent in CIMMYT's lowland tropical late and subtropical QPM germplasm (Vasal et al., 1993) and subtropical and temperate intermediate maturity germplasm (Beck et al., 1991).

The genetic interpretation of a diallel with a reduced number of parental inbreds, such as the ones in this study, can be biased by the lack of independent distribution of genes in the parental lines (Baker, 1978). Therefore, combining abilities reported here could be biased by the correlation of gene frequencies and should be interpreted with caution. Despite this limitation, these diallels were useful to determine which QPM inbreds had the most desirable expression of relevant traits and to estimate the heterotic relationship among them. The biplot analysis helped visualize graphically the best hybrid combinations and the relationship among the parental inbreds. A potential constraint of the biplot method is that it may not explain all of the variation (Yan and Hunt, 2002). The amount of variation explained by the two principal components was greater than 72% in both cases. In addition, the conclusions drawn from biplots were verified with the results from the conventional Griffing's analysis.

Both the white and yellow lines used in these diallels varied in adaptation (Table 2.1). CIMMYT and South African lines were mostly tropical and subtropical in adaptation whereas TAMU lines were more temperate adapted. The testing environments ranged in latitude from 26° N to 35° N representing a transition between subtropical and temperate areas of maize cultivation (Table 2.2). With increasing latitude and day length, QPM hybrids of subtropical lines tended to be late maturing with more

biomass, higher ear placement, and higher grain moisture content, as reflected by the GCA effects (Tables 2.5 and 2.7). Previous studies have shown that white QPM hybrids, in general, are more competitive for yield in subtropical environments as compared with temperate environments (Bhatnagar et al., 2003). Vasal (1993), in a 10-parent diallel study of tropical white QPM germplasm conducted in three environments in Mexico and USA, reported similar results. Overall, QPM hybrids yielded less than commercial checks. The gap in grain yield was greater in QPM yellow hybrids. Breeding efforts to enhance QPM hybrid performance in the USA should be devoted to increasing grain yield, standability, test weight, 1000-kernel weight, and to reducing ear placement, plant height, maturity, and grain moisture.

The classification of inbreds into heterotic groups facilitates the exploitation of heterosis in maize, which can contribute to hybrid performance. Vasal et al. (1993) reported information on the combining ability and heterotic patterns of CIMMYT's subtropical QPM germplasm. Recently, CIMMYT started classifying QPM inbreds into heterotic groups (HG "A" and HG "B") using two groups of testers (Cordova et al., 2003). Similar efforts have been undertaken in other breeding programs (e.g., TAMU). The biplot analysis in both white and yellow diallels suggests positive heterotic response between temperate and subtropical QPM inbreds that have been used as testers in these programs (Fig. 2.1 and 2.2). Therefore, inbreds from diverse backgrounds and adaptation can be used for hybrid identification and incorporation of exotic germplasm into temperate adapted inbreds for southern U.S. environments. Based on these results, it seems plausible to characterize and classify QPM inbreds into heterotic groups and to determine the relationship among groups used in temperate and exotic QPM lines. In these diallels, the best hybrids were formed between parental inbreds originating from different breeding programs, which suggest that these inbreds can produce high yielding hybrids. In future line recycling and in the development of source breeding populations, crosses among QPM lines from the same group may enhance the heterotic response as it has been observed in yellow dent maize.

The information obtained from these experiments can facilitate the identification of hybrids that combine quality traits, such as endosperm quality and disease resistance, from some inbreds with the adaptation and yield potential of other inbreds. For example, in the white hybrids, a breeding objective would be to combine endosperm hardness from TxX124, high test weight from CML176, low grain moisture from CML181, and reduced plant height and lodging, and early maturity from Tx807. In yellow hybrids, it would be desirable to combine high test weight from CML190, high yield from Tx802, and early maturity, low grain moisture, and low plant height from TxX808. A trait of particular interest in QPM is endosperm hardness because it is associated with large flaking grits and low dry matter losses in alkaline processing of maize, and also with lower incidence of insect and pest damage and grain aflatoxin at maturity (Betrán et al., 2002). Several modifier genes with additive gene action are involved in endosperm hardness in the *opaque 2* background of QPM (Wessel-Beaver et al., 1985; Vasal et al., 1993).

Superior QPM hybrids are extremely valuable for the white-maize food industry and yellow maize for feed in animal nutrition. In the USA, where almost 55% of corn produced is utilized as feed for swine and poultry, development of well-adapted QPM germplasm will have tremendous value for the feeding industry (Johnson et al., 2001). We concluded that the nutritional value of maize for both food and feed would be significantly enhanced by appropriate breeding strategies that emphasize the combination of desirable traits from exotic and temperate QPM lines.

CHAPTER III

AGRONOMIC, AFLATOXIN AND QUALITY ANALYSIS OF HIGH LYSINE MAIZE INBREDS AND TESTCROSSES

OVERVIEW

Quality Protein Maize (QPM) with the *opaque-2 (o2o2)* mutation is nutritionally superior to normal maize due to increased concentrations of essential amino acids lysine and tryptophan. QPM has hard endosperm texture due to the presence of modifier genes in the opaque-2 genetic background that make the maize kernel less susceptible to mechanical and biological damage. Texas A&M has developed a wide range of QPM inbreds by selecting and recycling several temperate high lysine inbreds and subtropical and tropical inbreds, populations and pools from other breeding programs (CIMMYT, México and University of Natal, South Africa). Agronomic evaluations of 48 inbreds derived from CIMMYT populations (65, 66, 69, 70) and pools (26, 33 and 34) in testcross combinations with Tx804 and checks were conducted across seven Texas locations. QPM inbreds in testcrosses have similar flowering dates, plant height, ear height and test weights but lower grain yield than normal checks. Testcrosses derived from QPM inbreds temperate x tropical high oil and populations 33 and 34 had comparable grain moisture content to normal checks. Repeatabilities on genotypic mean basis for grain yield were high in all seven environments (range from 0.73 to 0.92) and across all environments (0.67). The same testcrosses were evaluated for aflatoxin accumulation in a separate experiment in three environments in south and central Texas. Population 69 inbreds developed at CIMMYT that have a flinty orange texture were least susceptible to aflatoxin accumulation both in inbreds and testcrosses at all locations. Aflatoxin in testcrosses was positively correlated with endosperm texture (0.67) and kernel integrity (0.60) but negatively correlated with grain yield (-0.30) and silking date (-0.50). Quality evaluations of QPM germplasm to determine the levels of

amino acids tryptophan and methionine revealed negative correlations of tryptophan levels with endosperm translucence, a measure of kernel hardness. On average, germplasm containing the *o2/o2* mutation had lower methionine levels than *O2/O2* germplasm regardless of kernel hardness. Evaluations of inbreds testcrossed to Tx804 revealed a correlation of the amino acid levels of inbred lines with those of the hybrids, although the predictive value was low for methionine ($R^2 = 0.13$) and tryptophan ($R^2 = 0.27$). Selection for hard endosperm texture negatively impacts the nutritional value of QPM due to reduction in both tryptophan and methionine levels. Simultaneous selection for both these amino acids and hard endosperm types may enhance the nutritional value of QPM.

INTRODUCTION

Maize is the primary source of energy in animal rations in the USA and is being increasingly utilized as feed for animals in many developing countries, especially parts of Asia where total consumption of maize as feed exceeds 50% of total production (FAO, 2000). This spurt in maize consumption as animal feed is linked to increased consumption of meat in the developing countries which has increased almost three times as much as it did in the developed world in the last decade (Pinstrup-Andersen, et al., 1999). Most of this increased trend in meat consumption has occurred in Asia (Delgado et al., 1999). However, nutritional quality of maize protein is poor because of deficiencies of the essential amino acids lysine, tryptophan, and methionine (Glover and Mertz, 1987; Watson, 1988). Main reasons for poor quality of normal maize is the relatively high concentrations of prolamines or zeins storage proteins (50-60%) which are almost devoid of lysine and tryptophan, as compared to other storage proteins albumins (3%), globulins (3%) and glutelins (30-45%) that have a relatively higher lysine content of 5-6%, 5-8% and 4-5%, respectively (Paulis et al., 1975; Sodek and Wilson, 1971; Wilson, 1991). Lysine is the first limiting amino acid followed by tryptophan and threonine in the diets of swine and humans (Shimada and Cline, 1974).

Lysine could also be limiting in poultry diet if protein sources other than soybean meal are used (Johnson et al., 2001). Increasing the levels of these nutritionally limiting amino acids is an important objective of plant breeding programs. Discovery of the *opaque-2* gene (Mertz et al., 1964) and subsequent efforts by CIMMYT to develop maize inbreds having the *opaque-2* gene along with modifier genes that confer hard vitreous kernel texture and simultaneously selecting for superior agronomic characteristics have led to the development of Quality Protein Maize (QPM). Texas A&M has an extensive QPM hybrid and inbred line development program. Currently several high lysine inbreds having different levels of endosperm hardness and adaptation to temperate Southern U.S. growing conditions have been developed. Main approaches used for development of QPM inbreds are selection within the CIMMYT germplasm, conversion of standard U.S. inbreds to QPM, and recycling of QPM inbreds. Major emphasis of the program is adaptation to temperate U.S. conditions, normal kernel phenotype, increased grain yield, and aflatoxin resistance.

Preharvest aflatoxin contamination of corn is a chronic problem in southern U.S. and causes high economic losses annually to maize growers. Aflatoxins are also a serious risk to human and animal health causing liver cancer (Castegnaro and McGregor, 1998). There is immense variability in response and aflatoxin accumulation of maize hybrids, inbreds and cultivars but to date there has been no line reported to be completely resistance to aflatoxin under conditions conducive for disease. Limiting factors in breeding for aflatoxin resistance are the spatial and temporal variation in aflatoxin accumulation that requires inoculation and a high number of replications, the lack of a reliable and inexpensive screening methodology, and the low metabolic activity of corn plants after physiological maturity (Payne, 1992).

In general, hybrids or cultivars that are well adapted to the region of cultivation, having good husk cover and resistance to insects accumulate less aflatoxin (Payne, 1992; Betran and Isakeit, 2004). Since natural infection is undependable and variable, successful screening is dependent on reliable inoculation methods that significantly differentiate among the aflatoxin accumulation of testing genotypes. Several artificial

methods, including pinbar inoculation (inoculating kernels through the husk), non wounding silk channel inoculation (Zummo and Scott, 1989), and infestation of corn ears with insect larvae infected with *A. flavus* conidia, have been tried with varying degrees of success (Tucker et al., 1986; Windham and Williams, 1998).

Development of QPM at CIMMYT, México involved selection of genotypes resistant to ear rot and modified kernel texture while simultaneously improving agronomic characteristics. QPM germplasm represents a potential source of resistance to aflatoxin. White and yellow QPM hybrids have better husk cover, less ear rot, and less aflatoxin as compared with non-QPM commercial checks (Bhatnagar et al., 2003). The Texas A&M University maize breeding program has developed QPM inbreds adapted to temperate Southern U.S. growing conditions by selecting within CIMMYT QPM populations. The first set of inbreds with different backgrounds and origins is now available for testing for aflatoxin accumulation.

We had three major objectives in this study utilizing inbred lines developed from different QPM temperate adapted populations and their testcrosses:

(1) to evaluate testcrosses for grain yield and agronomic performance across Texas locations,

(2) to estimate aflatoxin accumulation and expression of associated traits, repeatibilities and correlations of these traits, and relationship between inbred lines and their testcrosses for aflatoxin accumulation,

(3) to characterize the variation in amino acids (tryptophan and methionine), effect of endosperm modification on tryptophan concentration, and estimate the relationship between inbred lines and their testcrosses for tryptophan and methionine levels.

REVIEW OF LITERATURE

Agronomic evaluation

Development and agronomic performance of quality protein maize

International Center for Maize and Wheat Improvement (CIMMYT), México in the early 1980's started a simultaneous conversion and population improvement program with multiple trait selection to accumulate modifiers, maintain improved protein quality, improved yield and resistance to ear rots and other agronomic traits. After several years of backcrossing-cum-recurrent selection most of CIMMYT's subtropical and tropical pools and populations were converted to QPM that had yields comparable to the normal commercial maize varieties. Later CIMMYT initiated a hybrid development program in 1985 with testing at locations worldwide in collaboration with National Agricultural Research Programs. Currently, CIMMYT is testing QPM hybrids, populations and inbreds in about 40 countries around the world. Excellent hybrids in both white and yellow QPM have been tested and released in many Latin American, African and Asian countries in the last three years. In 1999, CIMMYT evaluated superior subtropical and tropical QPM hybrids in more than 30 nations around the world. Results show that some of these hybrids had a yield advantage of one ton or more per hectare over the best normal maize hybrids (CIMMYT progress report, 1999). Results from Mexico showed the tropical x subtropical three-way cross hybrid [CML142 X CML150] X CML176 as one of the best hybrids yielding 8% more than the best normal commercial check. Another tropical white hybrid CML142 X CML146 in trials across 29 locations in Latin America, Asia and Africa yielded 6.7 t ha^{-1} in comparison to the normal commercial check that yielded 5.6 t ha^{-1}. Some yellow tropical hybrids with parents CML161 and CML172 have yielded consistently better over the normal commercial checks at locations worldwide (CIMMYT progress report, 1999). In 2003 CIMMYT has identified three promising QPM hybrids ([CML144 x CML159] x CML182, [CML144 x CML159] x CML181, and [CML140 x CML146] x CML143) for the mid-altitude zones

of Ethiopia, a maize streak virus resistant hybrid [CML144 x CML159] x CML176 for southern Africa, a new QPM hybrid [CML161 x CML493] for Vietnam, and released two new QPM hybrids in India. CIMMYT also released in 2003 two yellow and two white QPM inbreds of which CML491 was reported to have excellent GCA for yield, protein quality, and ear rot and foliar disease resistance (CIMMYT progress report, 2003).

Texas A&M in the early 1990's started evaluation of four white QPM hybrids selected from CIMMYT lines and some U.S. modified QPM lines. QPM hybrids were reported to out yield the best non-QPM commercial hybrid and in general had earlier flowering, excellent standability, good disease and insect resistance, and grain quality (Bockholt and Rooney, 1992). In the same study, yellow QPM hybrid (Tx802 x Tx814) was reported to have earlier silking, better standability and a yield of 0.13 tonnes more than the non QPM hybrid Conlee 202, however, the QPM hybrid had lower test weight and smaller seed size in comparison to the check hybrid. Bhatnagar et al. (2003) in evaluation of CIMMYT subtropical and tropical white and yellow QPM hybrids along with best commercial non QPM checks reported QPM hybrids to have bigger tassels, higher ear placements, longer flowering dates and in general lower yields across several Texas environments than non-QPM checks. In the same study both white and yellow QPM hybrids were significantly less susceptible to aflatoxin than non-QPM checks and had superior nutritional quality measured as average lysine per protein content (41.73 g kg^{-1} for white QPM vs. 34.13 g kg^{-1} for commercial checks and 41.91 g kg^{-1} for yellow QPM hybrids vs. 29.71 g kg^{-1} for non-QPM hybrids).

Most of the commercially grown temperate normal maize is represented by only a few elite lines (B14, B37, B73, B84, C103, Oh43, Mo17, and H99) (Troyer et al., 1988; Lu and Bernardo, 2001) that has resulted in limited genetic diversity mainly because of the recycling of elite inbreds by seed companies (Hallauer, 1990; Troyer, 1996). Introgression of desirable traits from exotic germplasm while practicing selection for adaptation to temperate growing conditions has been advocated to increase genetic diversity of temperate maize (Wellhausen, 1965; Michelini and Hallauer, 1993). Use of

tropical and subtropical germplasm has shown promise for introgression of desirable traits for quality and disease resistance that can be effectively utilized to broaden the genetic base of temperate maize and improve productivity (Goodman et al., 2000). Testcross evaluations of semiexotic inbreds derived from Latin American lines have shown lower grain yields in comparison to commercial hybrids but were competitive in grain moisture and lodging resistance (Tarter et al., 2003).

Aflatoxin evaluation

Aflatoxin contamination of maize grain

Aflatoxins are produced by the fungi *Aspergillus flavus* (B1 and B2) and *Aspergillus parasiticus* (G1, G2 and B1, B2). Both species are extremely diverse genetically and comprise large numbers of vegetative compatibility groups (VCGs), even within a restricted geographic area (Bayman and Cotty, 1991; Horn and Greene, 1995). Aflatoxins are highly toxic to livestock, poultry, and humans (Payne, 1998), particularly B1, which is the most carcinogenic and common aflatoxin in maize production in the U.S. and other parts of the world (Cullen and Newberne, 1994). Contamination of grain with mycotoxins causes accumulation of secondary metabolites that, if present in high concentrations, are extremely toxic resulting in death in humans, poultry and livestock. In lower concentrations it causes slow weight gain, or unthiftiness in animals and cancer of liver and esophagus in humans (Cheeke and Shull, 1985). In the U.S., aflatoxin contamination of maize has been reported in 23 states and is widely prevalent in the southeastern states where it has become a chronic problem (Payne, 1992; Widstrom, 1996). As per the standard set by the U.S. Food and Drug Administration (2000), aflatoxin contamination of maize grain intended for feed for immature livestock, dairy animals and food for humans should be less than 20 ng g^{-1}, for maize intended for feeding poultry less than 100 ng g^{-1}, for maize intended for feeding finishing swine less than 200 ng g^{-1} and for maize intended for finishing cattle should be less than 300 ng g^{-1}. Maize grain exceeding these levels cannot enter interstate

commerce and in general should not be fed to young livestock, lactating animals, or to animals that produce meat.

<u>Factors contributing to high pre-harvest aflatoxin contamination</u>

Contamination of temperate maize in the Corn Belt by *Aspergillus flavus* is considered primarily due to storage problems and can be prevented by proper bin sanitation and storage of grain. Preharvest field infection by *A. flavus* have been documented mainly in southeastern U.S.A. where drought stress and high ambient temperatures during kernel filling are conducive to growth of the fungi in the maize silk, colonization and subsequent aflatoxin infection in the maize kernel (Vincelli et al., 1995, Payne, 1998). The effect of irrigation in general has been reported to reduce *A. flavus* infection and aflatoxin concentration in maize (Jones et al., 1981). Insufficient uptake of nutrients associated with drought stress or leaching of mineralized N from the root zone due to excessive rain are also important factors (Jones, 1979). Deficiency of any essential plant nutrient increases the susceptibility of the plant to several plant pathogens (Stromberg et al., 1999).

Insect feeding activity has been found to be associated with fungal infection of maize grain and the subsequent production of mycotoxins in several ways such as, transport primary inoculum to the ears, move inoculum from the silks into the ears, disseminate inoculum within the ear, and facilitate colonization and infection of the kernels by injuring the kernels (Beti et al., 1995; Drepper and Renfro, 1990). Maize ears extensively damaged by European corn borer [*Ostrinia nubilalis* (Hubner)] and corn earworm [*Heliothis zea* (Boddie)] have been reported to show significantly higher levels of aflatoxin than undamaged ears (Lillehoj et al., 1975). McMillian et al. (1980) found that *A. flavus* sporulation and aflatoxin contamination increased in maize damaged by corn earworm and fall armyworm (*Spodoptera frugiperda*) feeding on the developing grain.

Management and control of pre-harvest aflatoxin contamination

Sound management practices can reduce mycotoxin contamination in the field such as timely planting of well adapted hybrids, adequate use of nitrogenous fertilizers (Jones, 1979), avoiding drought stress during kernel filling, controlling certain insect pests and proper harvesting (Lisker and Lillehoj, 1991; Miller, 2001). Duncan et al. (1979) in studies conducted over two years in North Carolina, found a negative correlation between maize grain yields and preharvest aflatoxin contamination. Good management practices such as effective weed control can reduce the incidence of aflatoxin by eliminating stress due to competition to the growing maize plant (Lillehoj, 1983). In Latin America, Africa and southern Asia incidence of mycotoxin contamination is more prevalent mainly due to lack of resources such as nitrogen, fertilizer, irrigation water, harvesting, transportation, handling and storage facilities. Unavailability of hybrids that are genetically more suitable to tolerate drought stress and susceptibility to insects and pests than open-pollinated cultivars in these countries aggravate the problem (Zuber et al., 1983).

Recently newer techniques such as biological control have been applied utilizing intraspecific competition between nonaflatoxigenic strain that occupy the same ecological niche as native aflatoxigenic strains and effectively compete with them during initial infection under conditions favorable for aflatoxin contamination (Cotty, 1994; Dorner et al., 1998). However, recombination in *A. flavus* through parasexual cycle between an introduced nonaflatoxigenic biological control strain and native aflatoxigenic strains in the field is a concern (Geiser et al., 1998). Competitive ability of strains can be affected by several other factors such as, enzyme production, growth rate, and capacity to survive in soil, that may be more important in determining the success of a biocontrol strain in inhibiting aflatoxin contamination within a crop (Horn et al., 2000).

Genetic control of resistance mechanism and breeding approaches to reduce pre-harvest
A. flavus contamination

Resistance to aflatoxin contamination is under genetic control (Scott et al., 1988; Widstrom et al., 1987). Aflatoxin has been associated with several plant traits that could influence or condition resistance to aflatoxin contamination and thereby affect the final aflatoxin accumulation such as husk coverage and tightness (McMillian et al., 1985; Lisker and Lillehoj, 1991; Odvody et al., 1997; Betrán and Isakeit, 2003), physical and chemical characteristics of the seed pericarp such as wax and cutin layers on maize kernels (Guo et al., 2001), drought and heat tolerance (Payne, 1992), resistance to insects (e.g., corn earworm) (Windham et al., 1999), kernel integrity (Odvody et al., 1997), maturity and adaptation to the local environments (Betrán and Isakeit, 2003), endosperm texture (Betrán et al., 2002), and resistance factors in kernels which reduce fungal development or aflatoxin formation (Brown et al., 2001). Genetically controlled enzymes or proteins have been identified that inhibit the growth of *A. flavus* in maize grain, such as an enzyme -1-3-Glucanase (Lozovaya, 1998), two proteins (100kDa and 28kDa) present in kernels of a resistant strain Tex 6 (Huang et al., 1997) and a 14kDa trypsin inhibitor in resistant genotypes (Chen et al., 1998). There is genetic variation for the expression of these traits and subsequently, for the response of maize hybrids to aflatoxin contamination (Scott and Zummo, 1992; Campbell and White, 1995; Betrán et al., 2002). Full-season hybrids have shown lower aflatoxin contents and better husk coverage than intermediate and early hybrids in Texas (Betrán and Isakeit, 2003). The correlation between silking date and aflatoxin accumulation was significant and negative (-0.59) and between husk cover and aflatoxin content was significantly positive (0.77).

Diallel mating designs to determine the genetics of resistance of *Aspergillus* ear rot have reported significant GCA effects but non-significant SCA effects and have shown additive genetic effects to be more important than dominance effects in the identified sources of resistance (Widstrom et. al., 1984). Studies conducted on combining genes for resistance to *Aspergillus flavus* with genes for resistance to corn earworm have shown two lines, GT-A1 and GT-A2, from population GT-MAS:gk and

Mp313E to be resistant (Guo et al., 1998). It was suggested that preharvest aflatoxin contamination can be controlled by pyramiding resistance genes from these lines into desirable elite lines through a backcross breeding program along with the use of marker assisted selection to accelerate the selection process. Several other inbred lines that are naturally resistant to aflatoxin have been identified such as inbreds Mp420, Mp715, Mp720, LB31, CI2, and Tex6 (Scott and Zummo, 1990; Campbell et al., 1993; McMillian et al., 1993; White et al., 1997).

Prediction of the response of a hybrid is complicated by many factors such as differences in environmental conditions, repeatability across different locations and years and a rapid and inexpensive method for measuring fungal infection and quantifying aflatoxin levels (Payne, 1992). Artificial inoculating techniques including kernel wounding to simulate insect injury, such as pinbar and razor blades have shown higher levels of aflatoxin levels (Calvert et al., 1978). Since kernel wounding techniques do not allow differentiation of genotypes on the basis of their natural attributes such as presence of aleurone layers or pericarp thickness have been replaced by non-wounding techniques such as silk channel inoculation (Jones et al., 1980; Zummo and Scott, 1989).

Heritabilities or repeatabilities for aflatoxin accumulation have been variable depending on the material tested and the number and type of environments. Betrán et al. (2002) in evaluations of inbred lines and hybrids under inoculation have reported repeatabilities at single locations higher than 0.50 when variation for aflatoxin accumulation was high. However, repeatabilities across locations have been generally low as a consequence of high genotype x environment interaction. The relationship between performance of inbred lines and their hybrids, and the degree of transmission to hybrids of the expression of traits in parental inbreds are important issues in hybrid development. Genetic correlation between parental inbreds and hybrids depends on the trait and in general is relatively high for some traits (e.g., plant morphology, ear traits, maturity, quality characters, etc.) but low for grain yield (Hallauer and Miranda, 1988). Hence, hybrid testing is required to identify the inbreds with the best breeding values. There is not much information about the type of relationship between inbreds and their

hybrids for aflatoxin accumulation. The amount of genetic variation among the inbreds tested and the type of tester may influence the correlation between line and testcross performance (Betran et al., 1997a). An aflatoxin resistant tester can reduce the correlation, likely due to favorable alleles masking effects of alleles present in lines, while a high susceptible tester can increase the genetic variation among testcrosses and the correlation.

Transgenic maize hybrids containing a gene from *Bacillus thuringiensis* (Bt) Beliner expresses an endotoxin, Cry1A(b) Bt protein, that have been useful in controlling lepidopteran insects in the larval stage (Williams et al., 1998) and observed to experience less *Fusarium* infection due to the association between insect feeding and the pathogen (Munkvold et al., 1997). Odvody and Chilcutt (2003) in a study of the effect of Cry1A(b) Bt gene on the amount and type of insect injury, preharvest aflatoxin content at maturity and agronomic performance of near-isogenic pairs (Cry1A(b) Bt/nonBt) of commericial maize hybrids have reported significantly higher whorl and ear injury by insects and greater aflatoxin content in nonBt hybrids as compared to the Bt hybrids. They reported a consistent significant positive correlation between aflatoxin content (log 10) and insect ear injury and a negative correlation of aflatoxin content with yield at all locations and years for the nonBt hybrids. Transgenic cotton has been reported to accumulate lower levels of aflatoxin contamination (Cotty et al., 1997).

Post-harvest control of aflatoxin contamination

Field drying of maize grain is common in Midwest and southeastern U.S.A. Harvesting maize grain having a moisture content in the range of 255 to 200 mg g^{-1} followed by artificial drying to 155 mg g^{-1}, usually within 14-28 days post physiological maturity has been recommended for safe storage or for transportation (U.S. Food and Drug Administration, 2000; Bruns and Abbas, 2001). Over drying grains results in kernel breakage causing the grain to become more prone to fungal infection by grain storage molds (Vincelli and Parker, 2001). Safe practices during harvesting (cleaning combines thoroughly prior and after harvest) and storage (cleaning bins thoroughly,

treatment with insecticides, separate storing of old and new grain, and blending at safe equilibrium relative humidity can greatly minimize aflatoxin contamination (Sauer and Burroughs, 1980).

Effects of nixtamalization in reducing aflatoxin toxicity to humans

Aflatoxin contamination of maize grain is a serious problem for the tortilla making industry, especially in México where maize is a staple food. Nixtamalization of maize meant for making tortillas is a process of steeping maize in lime solution and cooking until the grain becomes soft and can be easily divested of the pericarp. Nixtamalization has been reported to reduce the toxicity of grain with aflatoxin contamination greater than 50 ppb by 75 – 90% (Guzman-de-Peňa et al., 1995). Other studies have found an apparent decrease of aflatoxin by up to 46% evaluated under different processing conditions. However, acidifying nixtamalized products has also been reported to cause some reformation of the toxin and increased toxicity (Price and Jorgensen, 1985).

Detoxification of aflatoxins

Detoxification of aflatoxins (more than 80% of AFB) by the use of a phyllosilicate clay (hydrated sodium calcium aluminosilicate or HSCAS) mixed with feed has been shown with rats and chickens (Phillips et al., 1988; Phillips et al., 1991). In another study confirming the results, no new metabolites were identified in groups treated with HSCAS, suggesting that the AFB-HSCAS complex was not significantly dissociated in vivo (Sarr et al., 1995). Another method of detoxification of aflatoxin is through using chemical methods such as ammoniation and reaction with sodium bisulfite. Ammoniation involves the hydrolysis of the lactone ring and chemical conversion of the parent compound aflatoxin B1 to numerous products that exhibit greatly decreased toxicity. Sodium bisulfite reacts with aflatoxins (B1, G1, and M1) under various conditions of temperature, concentration, and time to form water-soluble products.

Quality evaluation

Maize endosperm protein characteristics

A typical maize kernel averages about 9.5% protein in the endosperm and 18% protein in the embryo on a 15.5 % moisture content basis. Maize endosperm proteins are categorized into two major types; storage proteins and nonstorage proteins. Prolamins or zeins are the most abundant storage proteins representing about 50% of the total proteins in mature seed (Soave et al., 1981) and 62-74% of the endosperm proteins (Landry et al., 2000; Hamaker et al., 1995). The other storage proteins comprise of glutelins (30-45%), albumins (3%), and globulins (3%). Zeins are specifically expressed during seed development being synthesized by membrane-bound polyribosomes and transported into the lumen of the endoplasmic reticulum where they are packaged in protein bodies during endosperm development (Larkins and Hurkman, 1978). Zein structural genes are represented by six "multigene families" that have been classified on the basis of their molecular weights into 4 distinct types, called alpha (19 and 22-kDa), beta (14-kDa), gamma (16 and 27-kDa) and delta (18 and 10-kDa) constituting 50-60%, 10-15%, 20-30% and 1-5%, respectively, of the total zein fraction in the maize endosperm (Larkins et al., 1984). All the four types of zeins aggregate to form protein bodies that are stably retained within membrane vesicles. The main function of zeins is to store nitrogen in the developing seed.

Amino acid composition of zeins

Zeins have a characteristic amino acid composition with almost 60% of total residues consisting of hydrophobic glutamine, proline, leucine and alanine and a very low level of basic amino acids, especially lysine (0.2 gm lysine/100gms). Almost all of the alpha zeins have been completely sequenced with the 19 and 22-kDa classes consisting of 210 and 245 residues, with true molecular weights of about 23,000-24,000 and 26,000-27,000, respectively (Coleman et al., 1995). The chains of the 22-kDa and 19-kDa proteins consist of several (9-10) serial, similar amino acid sequences. The

sequence analyses of alpha zeins have shown the presence of three unique domains (Pederson et al., 1982). The N-terminal domain consists of 36-37 residues and is followed by 10-11 tandem repeats having an average length of 20 residues and terminated by a C domain of 10 residues. The size difference between 19 and 22-kDa zeins is due to an additional repeat in the C-terminal end of the protein which results in a total of 10 repeats in the 22-kDa zein as compared to 9 in the 19-kDa zein (Song et al., 2001). The homologous domains are the products of gene sequences that originated by multiple duplication of a short original gene and that were subsequently combined in tandem to one gene. Numerous such cases have been described in animals. Zeins are impressing examples for the existence of this mechanism in plants.

Glutelins

Glutelins, are the next major source of lysine in maize endosperm protein (4-5%), and have been proposed as an alternative to selection for high lysine maize (Lin et al., 1997; Yau et al., 1998). Studies conducted at Texas A&M University on 29 inbred lines (Yau et al., 1998) revealed significant correlation between lysine content in six out of seven glutelin proteins (35, 43, 48, 84, 92 and 100 k-Da). The structure and amino acid sequence was determined of the maize endosperm *glutelin-2 gene* (Prat et al., 1985) and isolation and sequencing of a 28-kDa *glutelin-2* gene has been done (Boronat et al., 1986). Glutelins are a multigene family composed of 3 subunits, G1-204 (28-kDa), G1-164 (16-kDa), G2 (15-kDa) and G3 (10-kDa). Complete cDNA sequences of the four genes amplified by PCR to determine the exact number of copies per genome showed that G1 has 5-10 copies, G2 has 1-2 copies and G3 has 2-3 copies per genome (Lazzari et al., 1993).

Improvement in protein quality

Several mutants in maize were identified over the past 30 years *(opaque-2, floury-2, opaque-7, opaque-6, floury-3, mucronate, defective endosperm* and *opaque-11)* which alter the amino acid profile of the maize endosperm proteins elevating the levels

of deficient amino acids lysine and tryptophan at the expense of the most abundant zeins (Vasal, 2001). All these mutants vary tremendously in their inheritance characteristics, use and value in protein quality improvement (Vasal, 2001). The most widely studied and utilized mutant has been the *opaque-2*, a regulatory gene that reduces transcription of 22-kDa and 19-kDa fractions of the zein genes thereby causing a concomitant proportional increase in other lysine rich fractions (Moro et al., 1996; Shotwell and Larkins, 1989). Thus the lysine content of the maize kernel increases on an average by 40% making it more nutritively balanced (Moro et al., 1996). However, despite the nutritive advantages of the *opaque-2 gene*, it has been found to affect adversely some important agronomic characteristics and kernel phenotype of the maize plant such as reduced accumulation of dry matter, low grain yield, slower drying down, increased moisture content, lower weight and density of the kernel, poor kernel characteristics (soft, chalky and dull appearance) and greater susceptibility to ear rots and stored grain pests (Sreeramulu and Bauman, 1970; Vasal, 2001). In the past, several approaches to improve the protein content and kernel appearance of *opaque-2* mutant were explored including combining double mutants *opaque-2/floury-2* and *opaque-2/sugary-2* mutants (Vasal, 2001). Both approaches met with little success as the *opaque-2/floury-2* combination did not result in translucent kernels and the *opaque-2/sugary-2* combination resulted in a severe yield penalty of almost 20% over that of normal types. Another approach is to alter the germ-endosperm ratio that has the dual advantage of increasing both protein content and quality (Bjarnason and Pollmer, 1972; Poey et al., 1979; Vasal et al., 1980). The germ has almost double the amount of protein and is of better quality than the endosperm however, increasing the germ size contributes to poor shelf life of maize and will be an obstacle in some countries which throw away the germ before preparation of food products (Vasal, 2001).

Lysine and tryptophan content of *opaque-2* maize and QPM

Variability for lysine content in diverse maize genotypes has been documented in several studies (Bressani et al., 1962; Gevers, 1979). Percentage of lysine content in the

endosperm could be affected by three factors viz. the free amino acid pool, the zein proteins and the non-zein proteins. It has been shown that the free amino acid pool increases by 2-5 fold in opaque-2 germplasm as compared to normal, however the percentage of total non-protein lysine in the endosperm is only 5% (Lopes and Larkins, 1991). Lysine content has been shown to be positively correlated to the increase in the amount of non-zeins ($r^2 = 0.85$), and is not associated with the over production of free amino acids in the *opaque-2* germplasm (Moro et al., 1996). Determination of lysine content to distinguish high lysine maize inbreds and hybrids from normal maize is expensive and a severe limiting factor in most breeding programs (Mertz et al., 1974). Lysine levels have also been shown to be correlated with tryptophan levels, so rapid chemical methods to measure tryptophan are used to assess amino acid balance in plant breeding programs (Hernandez and Bates, 1969). The relationship between lysine and endosperm modification has been studied more thoroughly, and it has been determined that lysine levels in hard endosperm *o2* breeding germplasm were intermediate between *o2* and wild type germplasm (Ortega and Bates, 1983) and that lysine levels are negatively correlated with endosperm hardness (Wessel-Beaver et al., 1985). A study of several traits, including tryptophan levels and endosperm modification in QPM hybrids and open pollinated cultivars concluded that tryptophan levels and level of endosperm modification are not correlated, and that tryptophan levels are very stable across environments (Pixley and Bjarnason, 2002). In a study of tryptophan content in *o2*, modified endosperm *o2* and wild-type versions of inbred lines, the level of tryptophan was found to be reduced on average in the modified endosperm *o2* lines relative to the unmodified *o2* lines (Gentinetta et al., 1975).

Studies conducted to evaluate the effect of nitrogen fertilization on the nutritional value of grain protein in QPM and opaque-2 maize showed a linear increase in grain protein and lysine content of opaque-2 maize, however, protein content was observed to increase more rapidly than the lysine content as N fertilization increased that resulted in a net linear decrease of lysine expressed as a percentage of protein (Cromwell et al., 1983).

<u>Methionine content of normal maize</u>

Generally methionine is the first limiting amino acid in standard maize soybean diets for laying hens and young turkey (Bertram and Schutte, 1992; National Research Council, 1994). Increasing dietary methionine has been associated with greater egg production, higher egg weight (Harms et al., 1998) and increased efficiency of dietary protein utilization (Schutte, 1989). Attempts to identify maize inbreds high in methionine have been done in the past. Studies conducted on maize inbred line BSSS-53 that has high levels of kernel methionine (Phillips et al., 1981) have revealed a 10-kDa zein protein (Phillips and McClure, 1985) the structural gene for which designated as *dzs10*, is regulated posttranscriptionally by a *trans*-acting regulatory gene, *dzr1* which maps on chromosome 4 while the structural gene is located on chromosome 9 (Benner et al., 1989; Cruz-Alvarez et al., 1991; Chaudhuri and Messing, 1995). The *dzr1* BSSS53 allele is recessive to the *dzr1* Mo17 allele that accumulates low levels of 10-kDa zein (Schickler et al., 1993; Chaudhuri and Messing, 1994). Chaudhury and Messing (1991) showed increased expression of 10kDa zein in the line BSSS-53 to be responsible for its higher seed methionine content in comparison to other lines. The 18-kDa high-methionine delta-class zein gene from maize has been cloned, and its regulation, structure, and map position studied (Swarup et al., 1995). There studies have shown the 18-kDa and the related 10-kDa zein gene to be coordinately regulated, but their products accumulate to different levels in a genotype-dependent manner and also that zein genes may contain tryptophan and lysine codons.

<u>Transgenic approaches to increasing lysine content in maize</u>

Wallace et al. (1988) inserted Lysine (Lys) and Tryptophan (Trp) residues in different positions in the 19-kDa α-zein cDNA by nucleotide substitution and oligonucleotide insertion, later injecting the mRNA transcripts into frog oocytes, demonstrated that the modifications did not affect the synthesis, processing, stability, and deposition of the modified proteins into protein body oocytes and the Lys-rich α-zeins could also be produced in transgenic tobacco seeds like the normal zein. However,

the newly synthesized modified and normal zeins were both unstable and degraded (Ohtani et al., 1991). In another study Torrent et al. (1997) inserted Lys-rich (Pro-Lys)$_n$ residues in the Pro-Xaa region of the γ-zein and showed that the modified Lys-rich γ-zeins were accumulated to high levels in protein bodies and co-localized with the endogenous α- and γ-zeins in the transiently transformed maize endosperms. However, these mutated proteins were post-translationally modified in transgenic *Arabidopsis* plants, resulting in missorting and secretion to the leaf cell wall, while the normal γ-zeins were correctly targeted to the endoplasmic reticulum (ER) of the transgenic *Arabidopsis* leaf cells (Alvarez et al., 1998). Coleman et al. (1997c) transferred and expressed a mutant α-zein in maize that resulted in a *floury2* mutation phenotype in the seeds. Recently a transgenic maize line expressing a chimeric 10-kDa zein storage protein gene has been produced with enhanced mRNA stability through overcoming post-transcriptional regulation (Lai and Messing, 2002).

<u>Genetical and biochemical basis of selection for lysine content in high quality protein maize</u>

Major challenges facing breeders in successful introgression of multiple traits like *opaque-2* genotype and modifier genes while maintaining high lysine content are the expression of *opaque-2* gene only in the mature kernel and long duration in conversion of elite maize lines to QPM that usually requires at least 7-12 generations depending on the efficiency of backcrossing and selection. Several methods have been developed to facilitate genetic screening such as amino acid composition analysis, zein electrophoresis, enzyme-linked immunosorbent assay (ELISA) of alpha and gamma zeins (Wallace et al., 1990) chromatography analysis (Paulis et al., 1992) and dye binding capacity (Mossberg et al., 1969). However, due to the variable expression of the modifier genes in different backgrounds these techniques are not reliable and also delay the selection process until harvest. Recent molecular techniques such as DNA fingerprinting analysis utilizing genetic markers such as RFLP, AFLP, RAPD, SSR or SNP markers could obviate the need for testcrossing to identify the heterozygous

genotypes for backcrossing. An effective and accurate RFLP marker assay method of identifying plants with O2/O2, O2/o2 and o2/o2 genotypes from juvenile leaf DNA samples has been developed by the use of *Opaque-2* cDNA as a probe on HindIII-digested genomic DNA (Kata et al., 1994). Studies conducted on the association of other genes having effect on non-zein proteins such as EF 1-α, a lysine rich protein (10%) which binds the aminoacyl-tRNAs to the ribosome, has been found to be directly and significantly correlated to total lysine content of the endosperm protein that could facilitate indirect estimation of lysine content in maize endosperm (Habben et al., 1995). Other studies focused on the use of immunoassays based on glutelins, which are the next major source of lysine in maize endosperm protein (4-5%), as an alternative selection criteria have revealed significant correlation between lysine content in six out of seven glutelin proteins *viz.* 35, 43, 48, 84, 92 and 100-kDa (Lin et al., 1997; Yau et al., 1998). CIMMYT has initiated large scale fingerprinting of inbreds utilizing SSR molecular markers that are highly reliable and reproducible (Warburton et al., 2002).

To facilitate quick screening of high lysine germplasm a reliable method of estimating the relative contents of tryptophan and methionine has been proposed by Scott et al., (Maydica, *in press*) using a microbiological method suited to the high-throughput needs of plant breeding programs. In this method 10 mg of finely ground kernels are hydrolyzed in one-hundred microliters of 50 mM KCL adjusted to pH 2.0 with HCL containing 0.2 mg of pepsin. The reactions are then inoculated with a bacterial strain auxotrophic for either tryptophan or methionine in a suitable media. After incubation, the concentrations of amino acids are measured by scattering 595 nm light in a microplate reader.

Food and feed properties of high quality protein maize

Studies conducted to compare the wet milling properties of QPM and regular dent maize with contrasting endosperm textures have reported QPM and feed maize to have higher water solubility and faster water penetration than normal food maize, presumably due to less dense and softer endosperm texture (Gomez et al., 1992). Wet

milling properties of QPM evaluated on the basis of yields of germ, fiber, starch and gluten have shown QPM to compare favorably to that of food and feed maize. In addition to having high quality protein, QPM lines were found to have higher amount of fat in the germ, bran and gluten with almost equal amounts of starch in comparison with the food and feed maize. These observations suggest that they can economically replace regular maize in wet milling processes (Gomez et al., 1992).

A comparison of yield from dry-milled fractions of five QPM samples compared with three normal dent maize revealed higher yields of total grits and prime products (total grits + low fat meal + low fat flour) for QPM in comparison with the dent maize (Wu, 1992). Also QPM could be easily degermed and roller milled normally thereby indicating their suitability in producing dry milled products of high nutritive value. Studies on alkaline processing properties of white and yellow QPM indicate that excellent tortillas and chips can be made with slight alteration in the cooking conditions due to smaller kernel size (Sproule et al., 1988). QPM tortillas and tortilla chips fried from QPM have been reported to possess excellent flavor, rollability, color and retained higher amounts of dietary fiber due to incomplete pericarp removal with a shelf life comparable to that of normal maize chips (Serna-Saldivar et al., 1992).

In animal feed trials conducted to evaluate the nutritional value of QPM, pigs fed with QPM based diet had a higher utilization of feed and grew faster than pigs fed food and feed maize diets containing the same level of soybean meal supplementation (Knabe et al., 1992). Nitrogen retention expressed as percent N absorbed, was also highest (P < 0.05) for the same pigs fed with QPM diets, presumably due to higher content of lysine and tryptophan.

MATERIALS AND METHODS

Agronomic evaluation

<u>Germplasm</u>

A total of forty-eight inbreds and their testcrosses with Tx804 (Appendix B, Table B.1), a soft endosperm *o2* temperate inbred, were evaluated in seven locations in south and central Texas: College Station (CS), Weslaco (WE), Granger (GR), Bardwell (BA), Castroville (CA), Wharton (WH) and Springlake (SP) during year 2002. The inbreds were developed from the following CIMMYT QPM populations: 2 from Population 65 (yellow flint), 2 from Population 66 (yellow dent), 16 from Population 69 (temperate yellow flint), 7 from Population 70 (temperate yellow dent), 2 from Pool 26 (tropical late yellow dent), 2 from Pool 33 (subtropical intermediate yellow flint), 3 from Pool 34 (subtropical intermediate yellow dent), and 14 from Temperate x Tropical High-Oil population. These lines represent a group of yellow QPM lines selected for temperate adaptation. They were advanced and selected in Texas maize nurseries (summer nursery at College Station, TX, and fall-winter nursery at Weslaco, TX) based on endosperm modification, maturity, grain color, grain yield, lodging, lysine content and plant characteristics. Three inbreds Tx802 (Betran et al., 2003e), CML161 and Tx804 were used as checks in the inbred line evaluation. Five hybrids Pioneer Brand 31B13 and 32R25, Dekalb DK668 and DK667, and Asgrow RX897 were used as commercial checks in the testcross trial. In addition, three high lysine hybrids from Crow's Hybrid Company, SR470, SL53 and SR660, and 4 QPM hybrids (Do940y/Tx804, CML161/Tx804, (Do940y/Tx802)/Tx804, and Do940y/Tx802) were added to the experiment to complete a sixty entry trial.

<u>Field management and measurements</u>

Experiments at all environments were planted following alpha-lattice design (Patterson and Williams, 1976) with 2 replications. Characteristics of individual

environments are presented in Appendix B, Table B.2. Standard cultural and agronomic practices were followed at each environment. Data were recorded on a plot basis on the following agronomic traits: grain yield (combine harvested grain weight expressed in Mg ha^{-1} and standardized to 155 g kg^{-1} moisture content), silking date (number of days from planting until 50% of the plants showing silks), plant height (distance in cm from the ground to the top of tassel), ear height (distance in cm from the ground level to the main ear-bearing node), root lodging (% plants leaning at an angle greater than 30% from the vertical), stalk lodging (% plants with broken stalks at or below the main ear at maturity), grain moisture (g kg^{-1} moisture of grain at harvest), and test weight (recorded as g pint^{-1} by standard equipment and converted to kg m^{-3}).

Statistical analysis

Analyses of variance and adjusted means were derived for each experiment following SAS procedures GLM and MIXED (SAS, 1997). Adjusted means that had the lowest standard errors were used for calculating means across locations. Entry means were estimated considering genotypes (testcrosses or inbreds) fixed effects and replications and environments as random effects. Repeatability (genotypic-mean basis) for individual experiments was calculated as:

$$R = \frac{V_g}{V_g + \dfrac{\sigma_e^2}{r}}$$

where, V_g is the variance of differences among genotypic means, σ_e^2 is the error variance, and r is the number of replications. Broad sense repeatability estimates across environments were calculated as:

$$R = \frac{V_g}{V_g + \dfrac{\sigma_{ge}^2}{e} + \dfrac{\sigma_e^2}{re}}$$ where, V_g, σ_e^2 and r are same as above, σ_{ge}^2 is genotype x

environment interaction variance and e is the number of environments. Since inbreds were selected during their development and do not represent a population, therefore

repeatabilities instead of heritabilities were estimated. Pearson phenotypic correlation coefficients of grain yield with associated traits were computed for inbred and testcrosses at individual environments and across environments.

Aflatoxin evaluation

Germplasm

Sixty entries including 48 QPM inbreds with their testcrosses with Tx804 as described in the agronomic evaluation section previously, were evaluated in three locations in south and central Texas: College Station (CS), Weslaco (WE), and Corpus Christi (CC) during year 2002. Four non-QPM commercial hybrids, Pioneer Brand 31B13 and 32R25, Dekalb DK668 and Asgrow RX897 were used as checks in the testcross trial. QPM hybrids were the same as evaluated before in the agronomic trials except (CML 161 x CML 170) that was added to complete a sixty entry trial. The checks were commercial hybrids with different response to aflatoxin in previous evaluations.

Maize inoculation and aflatoxin quantification

Aspergillus flavus isolate NRRL3357, colonizing autoclaved maize kernels, was placed on the soil surface between treatment rows when the first hybrids reached mid-silk stage. The inoculum was distributed at the rate of 1 kg (noncolonized dry seed equivalent) per 200 foot of row (Odvody, personal communication). All the ears in the plot at three locations were hand harvested after kernel moisture in all hybrids was below 150 g kg^{-1}. Ears were husked, rated for insect injury and visible fungi colonization, dried, and shelled. The kernels were bulked within plots. The whole kernel samples were ground using a Romer mill (Union, MO). Quantification of aflatoxin was conducted in 50 g subsamples from each plot with monoclonal antibody affinity columns (Aflatest) and fluorescence determination using the Vicam fluorometer system (Watertown, MA). Aflatoxin content was expressed in ng g^{-1} (i.e., ppb).

Field management and measurements

Alpha-lattice field experimental design with 4 replications at CS and WE, and 3 replications at CC was used. Plots at CS and WE were 6.40 m long and 0.75 m apart, and 7.90 m long and 0.96 m apart at CC. No insecticides were applied after planting. Trials were planted at regular times in WE (middle of February) and CS (early March). Drought and heat stress was induced by late planting at CC (4 weeks later than usual planting time, which is the middle of February), and by limited irrigation at WE and CS. In addition to aflatoxin content, the following secondary traits were measured: grain yield (hand harvested dried grain weight expressed in Mg ha^{-1}), female flowering (FF) as days from planting to 50% of the plants in one plot with emerged silks, visual rating for kernel integrity (1= all ears without splits kernels or damaged by insects to 5 = most of the ears with splits and/or insect damage), endosperm texture as visual rating from 1 (flint = round crown kernel and vitreous appearance) to 5 (dent = kernels with pronounced dentation and high proportion of floury endosperm).

Statistical analysis

Aflatoxin contents in ng g^{-1} were transformed to log (ng g^{-1}) to equalize variance. This transformation is commonly used for aflatoxin contents. Both logarithmic and untransformed aflatoxin values were used in the analysis and presentation of results. Individual analyses of variance and computation of means were conducted for each experiment following GLM and MIXED procedures from SAS (SAS, 1997), and with AS-REML software that contains spatial analysis (Gilmour et al, 1997). Adjusted means calculated applying spatial analysis were the ones with the lower standard error and they were used in all the subsequent calculations. To estimate entry means, genotypes (testcrosses or inbreds) were considered fixed effects.

Repeatability on a genotypic-mean basis was calculated in the same way for individual experiments as described in the agronomic evaluation, except that in the calculation of broad sense repeatability estimates across environments, *r'* the harmonic mean of replications was used instead of r.

Pearson phenotypic correlation coefficients among aflatoxin and associated traits were computed for inbred and testcrosses at individual environments and across environments. To estimate the relationship between inbreds and testcrosses simple regression of testcross means across locations on means for inbreds at Weslaco for aflatoxin accumulation, grain yield, silking date, endosperm texture, and kernel integrity was computed using REG procedure in SAS (SAS, 1997).

Quality evaluation

Germplasm

Evaluations were conducted on three sets of germplasm with each set containing about 80 accessions (Appendix C, Tables C.1-C.3): (1) Inbreds 1: QPM lines developed from CIMMYT QPM populations 65, 66, 69, 70 and Temperate x Tropical High-Oil, and QPM lines from CIMMYT Pools 26, 33, and 34 (2) Testcrosses: Inbreds in set 1 crossed with Tx804 a soft endosperm $o2/o2$ inbred, and (3) Inbreds 2: Opaque-2 ($o2/o2$) soft endosperm lines developed from Crow's Seed Company segregated populations that are classified as Iowa Stiff Stalk Synthetic (SS) or non Stiff Stalk (NSS) groups, and QPM lines with hard endosperm texture that were advanced and selected in Texas nurseries based on endosperm modification, maturity, grain color, grain yield, lodging, lysine content and plant traits. High lysine inbreds (Tx802, CML161, Do940y, B73 $o2/o2$, and Tx804), and normal inbreds (Tx772, NC300, FRB73, FR2128, B104, and Tx601y) were included as checks in the inbred line evaluations. Commercial hybrids Crow's SL53, Pioneer Brand 31B13 and 32R25, Dekalb DK668 and DK687, and Asgrow RX897 were included as checks in the evaluation of testcrosses.

All three sets of germplasm were advanced in single plots at Texas A&M University during the summer of 2002 at CS, except for grain used in analyzing the relationship of Trp and Lys, which was produced in 2001 at CS. Plots were irrigated and fertilized with 350 kg ha^{-1} of 32-0-0 and 6 units of Zn before planting and 180 kg ha^{-1} of 32-0-0 at V6 stage. All ears in a line or testcross used in this study were self pollinated

by hand, harvested, bulked within genotypes, and the grain dried to approximately 12% moisture. Endosperm modification ratings (opaque = 1, semi-vitreous = 3, and vitreous translucent = 5) for 50 kernels per genotype were visually measured on a light box using a weighted average per genotype.

Quantification of amino acids

Lysine was quantified using the AOAC standard method for determination of lysine levels in grain (AOAC, 1990). Separation and analysis of amino acids were done with a Beckman 6300 Amino Acid Analyzer (Elk Grove, GA) equipped with a high performance cation-exchange resin column, and amino acid detection was done with a post-column ninhydrin derivation. Norleucine was used as the internal standard.

Tryptophan and methionine were quantified using a microbiological method described by Scott et al. (Maydica 2004, *in press*) as follows: kernels from bulked ears were finely ground, mixed and 10 mg of the resulting powder was weighed into a randomly assigned well of a 96-well plate. Ten wells were not filled to accommodate standards. In order to extract and hydrolyze protein in the ground grain, one-hundred microliters of 50 mM KCL adjusted to pH 2.0 with HCL containing 0.2 mg of pepsin were added to each well and the plate was shaken 16 hours at 37°C. The plate was then centrifuged at 3000 x g for 20 minutes, and 4 μl of the supernatant for methionine analysis or 7 μl of the supernatant for tryptophan analysis was transferred to the corresponding well of a second plate for analysis. Five microliters of standards consisting of commercially obtained (Sigma, St. Louis, Mo) methionine or tryptophan in the concentrations of 0.1 to 0.8 mM and 0.1 to 0.6 mM, respectively, were added to the empty wells of the plate. This plate was inoculated with a bacterial strain auxotrophic for either tryptophan (CAG 18455, Singer et al., 1989) or methionine (P4X, Jacob and ollman, 1961) in 100 μl M9 minimal media. This plate was incubated with shaking at 37°C for 20 h for tryptophan analysis or 16 hours for methionine analysis. Following incubation, the 595 nm light scattered by the culture was measured in a microplate reader.

Statistical methods

Each ground sample was analyzed in three replications (3 independently randomized 96-well plates) following a randomized complete block design with each plate representing a block. The methionine and tryptophan concentration in each analysis was calculated using linear regression onto a line fitted to the standards. The predicted value of each sample was calculated from the three individual measurements using a linear ANOVA model with the plate considered a fixed effect. Relationship between amino acid levels on inbreds and their testcrosses was computed using simple regression of testcross means on means for parental inbreds following the REG procedure in SAS.

RESULTS AND DISCUSSION

Agronomic evaluation

Agronomic performance

The results for agronomic performance of testcrosses between high lysine inbreds with different origins and Tx804 and hybrid checks across all locations are presented in Table 3.1. Significant differences in flowering dates were observed across all locations that varied according to the origin of testcrosses, QPM, opaque and normal checks. Testcrosses of opaque inbreds flowered almost 4 days earlier (71.03) than the average flowering date across all locations (73.80 days), whereas testcrosses of QPM inbreds flowered almost 3 days later than average (76.38 days). Testcrosses of populations 69 and 70, pools 33 and 34, temperate x tropical high oil and normal checks had intermediate flowering dates. Average plant height and ear height across all locations were 213.88 cm (range from 204.92 to 222.19 cm) and 80.48 cm (range from 74.13 to 87.38 cm), respectively. Testcrosses derived from population 70, pool 33 and 34 and temperate x tropical high oil showed significantly shorter plant heights and lower ear placements than QPM and normal checks that were taller and had higher ear placements. QPM inbreds CML 161 developed at CIMMYT, Mexico and Do940y

Table 3.1. Means and their standard errors for agronomic traits of testcrosses between high lysine inbreds with different origins and Tx804 and hybrid checks across all locations in 2002.

Hybrid Origin	Number of Hybrids	Grain Yield	Silking Date	Plant Height	Ear Height	Root Lodging	Stalk Lodging	Grain Moisture	Test Weight
		$Mg\ ha^{-1}$	days	cm	cm	%	%	$g\ kg^{-1}$	$kghl^{-1}$
P65 and 66	4	6.28±0.18	75.20±0.39	219.76±2.63	83.61±1.57	1.72±0.23	37.10±0.23	156.00±1.14	76.82±0.52
P69	16	6.35±0.09	73.94±0.20	219.18±1.32	82.88±0.79	1.93±0.11	52.25±0.11	154.72±0.57	76.39±0.26
P70	7	5.65±0.14	73.45±0.30	204.92±1.99	77.82±1.19	2.17±0.17	32.38±0.17	145.72±0.87	75.34±0.39
P26	2	5.77±0.26	74.27±0.55	219.92±3.72	82.62±2.22	2.85±0.32	35.68±0.32	155.04±1.62	74.47±0.74
P33 and 34	5	5.37±0.16	73.77±0.35	207.25±2.35	74.13±1.41	2.09±0.20	33.65±0.20	143.60±1.02	72.77±0.47
Temp. x Trop. High-Oil	14	5.37±0.10	72.90±0.21	206.70±1.41	76.22±0.84	2.38±0.12	28.55±0.12	138.83±0.61	73.20±0.28
QPM	4	6.69±0.18	76.38±0.39	224.31±2.63	87.38±1.57	1.98±0.23	38.94±0.23	164.58±1.14	75.18±0.52
Opaque Crow's	3	7.11±0.21	71.03±0.45	210.21±3.04	79.75±1.82	2.06±0.26	36.17±0.26	111.28±1.32	68.71±0.60
Checks	5	8.71±0.16	74.60±0.35	222.19±2.35	86.45±1.41	1.54±0.20	46.89±0.20	132.94±1.02	75.68±0.47
Means across locations	60	6.20±0.05	73.80±0.10	213.85±0.68	80.48±0.41	2.07±0.06	39.15±0.06	145.77±0.30	74.66±0.13
LSD for all 60 entries		0.59	1.72	11.02	7.40	1.13	27.82	6.79	2.42
CV (%)		4.83	1.18	2.61	4.67	27.70	35.89	23.69	1.65
Correlation with GY			0.23	0.48	0.55	-0.39	-0.10	-0.12	74.52
Min		5.37	71.03	204.92	74.13	1.54	9.78	111.28	68.71
Max		8.71	76.38	224.31	87.38	2.85	70.84	164.58	76.82

Table 3.2. Mean grain yield, repeatabilities and their standard errors of testcrosses per location and across locations in QPM inbred and their testcrosses in 2002.

Hybrid Origin	Number of hybrids	CS*	WE	CA	WH	GR	BA	SP	Across
		....Mg ha^{-1}..							
Pop 65 and 66	4	9.27±0.42	5.63±0.16	7.39±0.20	6.05±0.40	5.44±0.20	6.62±0.14	3.60±0.32	6.28±0.18
P69	16	9.01±0.21	5.90±0.08	7.67±0.10	5.30±0.20	5.47±0.10	7.07±0.07	4.03±0.16	6.35±0.09
P70	7	7.77±0.32	4.76±0.12	7.14±0.15	4.58±0.31	4.76±0.15	6.04±0.10	4.48±0.24	5.65±0.14
PO26	2	9.44±0.60	4.84±0.23	7.32±0.29	3.35±0.57	4.98±0.28	6.47±0.19	3.96±0.45	5.77±0.26
Pool 33 and 34	5	8.03±0.38	4.65±0.14	6.08±0.18	4.02±0.36	3.96±0.18	6.16±0.12	4.72±0.28	5.37±0.16
HO	14	7.11±0.23	4.14±0.09	6.62±0.11	4.30±0.22	4.04±0.11	6.30±0.07	5.13±0.17	5.37±0.10
QPM	4	10.43±0.42	5.50±0.16	8.03±0.20	6.45±0.40	5.44±0.20	6.29±0.14	4.72±0.32	6.69±0.18
Opaque Crow's	3	8.74±0.49	5.91±0.19	8.05±0.23	6.68±0.47	5.13±0.23	7.15±0.16	8.10±0.37	7.11±0.21
Checks	5	12.01±0.38	8.15±0.14	9.67±0.18	8.75±0.36	6.72±0.18	8.17±0.12	7.48±0.28	8.71±0.16
Mean	60	8.72±0.32	5.36±0.12	7.42±0.15	5.36±0.31	5.01±0.15	6.70±0.10	4.94±0.24	6.20±0.05
LSD for all 60 entries		2.42	1.09	1.19	2.34	1.17	0.76	1.82	0.59
Min		7.11	4.14	6.08	3.35	3.96	6.04	3.60	5.37
Max		12.01	8.15	9.67	8.75	6.72	8.17	8.10	8.71

*CS=College Station, WE=Weslaco, CA=Castroville, WH=Wharton, GR=Granger, BA=Bardwell, SP=Springlake

developed at University of Natal, South Africa are both subtropical in origin and consequently show late maturities, are tall with high ear placements in more temperate Texas environments. Bhatnagar et al. (2003) in an evaluation of subtropical and tropical QPM hybrids have reported similar results. No significant differences were observed in root and stalk lodging across locations among different testcrosses, QPM and normal checks. Significant differences for grain moisture content were observed among different testcrosses, QPM and normal checks. Grain moisture across locations averaged 145.77 g kg^{-1} with a range of 111.28 g kg-1 for temperate opaque inbreds to 164.58 g kg^{-1} for QPM inbreds. Testcrosses derived from populations 65, 66, 69 and pool 26 had significantly higher grain moisture content, whereas the checks had significantly lower grain moisture content than average. Test weight across locations averaged 74.66 kg hl^{-1} ranging from 68.71 kg hl^{-1} for opaque inbreds that have more floury endosperm texture to 76.82 kg hl^{-1} for testcrosses derived from populations 65, 66 and 69 that have flinty and hard endosperm texture.

Grain yield across locations varied significantly for all testcrosses between high lysine inbreds with different origins and Tx804 and hybrid checks and averaged 6.20 Mg ha^{-1} (Table 3.2). Average grain yield for hybrid checks across locations was 8.71 Mg ha^{-1} as compared to opaque-2 testcrosses (7.11 Mg ha^{-1}). Average grain yield across locations for QPM testcrosses varied from 5.37 Mg ha^{-1} for pools 33, 34 and temperate x tropical high oil testcrosses to 6.69 Mg ha^{-1} for testcrosses with TAMU QPM inbreds. Average grain yield per location ranged from 8.72 Mg ha^{-1} for environment CS to 4.94 Mg ha^{-1} for environment SP. In general, normal checks yielded highest followed by opaque and QPM, both per location and across locations.

The testing environments in this experiment ranged in latitude from 26° N to 35° N representing a transition between subtropical environments in southern Texas to more temperate environments of maize cultivation in U.S.A. Subtropical hybrids and inbreds originating in CIMMYT, Mexico and University of Natal, SA are less adapted to temperate environments of U.S.A. and in general mature later, grow taller and have higher grain moisture content (Bhatnagar et al., 2003). However, QPM inbreds

developed in Texas from populations 65, 66 and 69 showed proper maturity, were flinty hard endosperm types and had high test weights and endosperm quality that are useful traits for introgression into temperate normal germplasm. It has also been observed that inbreds from CIMMYT and University of Natal, SA have excellent combining abilities with temperate Texas inbreds (Bhatnagar et al., 2004). Breeding efforts directed to characterize further the heterotic pattern of inbreds derived from high lysine populations and pools included in this experiment can combine excellent grain quality traits of these germplasm with high yield, standability and early maturity of temperate germplasm.

Repeatabilities on genotypic mean basis for grain yield and agronomic traits are presented in Table 3.3. In general repeatabilities for grain yield were high ranging from 0.73 for environment Wharton to 0.92 for environment Weslaco. Repeatability across environments was moderately high (0.67). The inbreds included in this experiment varied greatly in their origin, subtropical hard flinty types (population 65, 66 and 69), temperate x tropical high oil, QPM, opaques from Crow's hybrids Co., and normal corn belt dent commercial checks. As such high genetic variability in these experimental inbreds could be the cause of high repeatabilities observed for grain yield. Repeatabilities for plant height and ear height were low for environment CS (0.44, 0.09) and across all locations (0.45, 0.33) but moderately high for other environments. Repeatabilities for silking date were moderately high for environments WE (0.65), WH (0.70) and across all locations (0.57). For grain moisture repeatabilities were high for all environments except GR (0.52). Repeatabilities for test weight were high for most environments except for WE (0.40), GR (0.26) and across all locations (0.45).

Table 3.3. Family based repeatabilities and their standard errors for grain yield and other agronomic traits per location and across locations of QPM testcrosses between high lysine inbreds with different origins and Tx804 and hybrid checks.

Traits		CS	WE	CA	WH	GR	BA	SP	AC
Grain Yield	Mg ha^{-1}	0.79±0.06	0.92±0.03	0.86±0.04	0.73±0.08	0.83±0.05	0.89±0.03	0.80±0.06	0.67±0.05
Plant Height	cm	0.44±0.15	0.78±0.07	-	-	0.77±0.07	-	0.60±0.11	0.45±0.07
Ear Height	cm	0.09±0.24	0.78±0.07	0.65±0.10	-	-	-	0.74±0.07	0.33±0.06
Silking Date	days	-	0.65±0.09	-	0.70±0.09	-	-		0.57±0.08
Root Lodging	%	-	-	-	0.21±0.21	0.35±0.17	-		0.13±0.08
Stalk Lodging	%	-	-	-	-	-	-	0.60±0.10	-
Grain Moisture	g kg^{-1}	-	0.96±0.01	0.97±0.01	0.91±0.03	0.52±0.12	0.76±0.07	0.95±0.01	0.71±0.05
Test Weight	Kg hl^{-1}	0.81±0.06	0.40±0.16	0.86±0.04	0.85±0.04	0.26±0.19	0.99±0.01	0.92±0.02	0.45±0.06

Aflatoxin evaluation

<u>Aflatoxin and plant traits in inbreds and testcrosses</u>

Significant differences were detected for all traits in both inbreds and testcrosses. Aflatoxin accumulation in inbreds at WE averaged 286.3 ng g^{-1} ranging from 12.5 ng g^{-1} to 2133.0 ng g^{-1} (Table 3.4). Grain yield for inbreds was relatively low (0.4 Mg ha^{-1} average) due to severe drought stress induced in the experiment. The levels of aflatoxin contamination for testcrosses averaged 596.78 ng g^{-1} at CC ranging from 268.5 to 2063.2 ng g^{-1}, 325.12 ng g^{-1} at WE ranging from 85.2 ng g^{-1} to 948.2 ng g^{-1}, and 105.72 ng g^{-1} at CS ranging from 75.4 to 229.5 ng g^{-1} (Table 3.5). These levels of aflatoxin contamination at CC and WE are similar or relatively higher compared with other studies (Betrán et al., 2002; Widstrom et al., 1984; Scott and Zummo, 1988). The ground inoculation with colonized kernels was effective to expose maize ears to *A. flavus* particularly in these two locations by increasing the amount of inoculum available for natural infection.

The response to aflatoxin and the expression of associated traits such as endosperm texture and kernel integrity was significantly different among the groups of inbreds based on their origin. Testcrosses of inbreds derived from Population 69 were most resistant to aflatoxin accumulation both in inbreds and testcrosses at all locations (Table 3.4 and 3.5, Fig. 3.1). These Population 69 inbreds have flinty endosperm, orange grain color, intermediate maturities, and dark green leaves. Testcrosses of inbreds derived from Temperate x Tropical High-Oil population and opaque commercial hybrids SR470, SL53 and SR660 showed highest aflatoxin accumulation. Larger embryo size and higher oil content of these inbreds, as compared with the rest of the inbreds, could be a factor associated with greater aflatoxin accumulation. *Opaque-2* mutants were more susceptible to aflatoxin contamination and infection by *A. flavus* in another study

Table 3.4. Means and standard errors for aflatoxin content and associated traits in inbreds with different origins at Weslaco, TX in 2002.

Inbred Origin	Number of inbreds	Aflatoxin	Aflatoxin	Grain Yield	Endosperm Texture	Kernel Integrity	Silking date
		log ng g^{-1}	ng g^{-1}	Mg ha^{-1}	1 to 5	1 to 5	days
				QPM Inbreds			
P65&66	4	1.2 ± 0.19	128.0 ± 138.4	0.3 ± 0.08	1.2 ± 0.11	3.0 ± 0.12	81.4 ± 0.34
P69	16	0.9 ± 0.09	113.1 ± 69.2	0.4 ± 0.04	1.1 ± 0.06	2.7 ± 0.06	81.4 ± 0.17
P70	7	1.9 ± 0.14	238.1 ± 104.6	0.7 ± 0.06	1.3 ± 0.08	2.8 ± 0.09	80.2 ± 0.26
P26	2	1.8 ± 0.27	165.2 ± 195.8	0.3 ± 0.11	1.9 ± 0.16	3.2 ± 0.17	81.7 ± 0.48
P33&34	4	1.5 ± 0.19	193.6 ± 138.4	0.3 ± 0.08	1.4 ± 0.11	3.0 ± 0.12	80.3 ± 0.34
Temp. x Trop. High-Oil	14	2.1 ± 0.10	612.9 ± 74.0	0.5 ± 0.04	2.6 ± 0.06	3.7 ± 0.07	79.6 ± 0.18
				Checks Inbreds			
CML161	1	0.6 ± 0.38	158.4 ± 276.9	0.3 ± 0.15	1.2 ± 0.22	2.5 ± 0.24	82.8 ± 0.68
Tx802	1	2.3 ± 0.38	321.5 ± 276.9	0.3 ± 0.15	2.0 ± 0.22	4.3 ± 0.24	81.0 ± 0.68
Tx804	1	1.7 ± 0.38	161.8 ± 276.9	0.5 ± 0.15	2.9 ± 0.22	4.2 ± 0.24	80.3 ± 0.68
				Mean of All Inbreds			
All inbreds	50	1.5 ± 0.05	286.3 ± 39.2	0.4 ± 0.02	1.7 ± 0.03	3.1 ± 0.03	80.1 ±0.10

Table 3.5. Means and standard errors for aflatoxin content and associated traits of testcrosses between high lysine inbreds with different origins and Tx804 and hybrid checks across three Texas locations, and means at single locations in 2002.

Hybrid Origin	Number of hybrids	Trait					
		Aflatoxin	Aflatoxin	Grain Yield	Endosperm Texture	Kernel Integrity	Silking date
		$\log$ ng g^{-1}	ng g^{-1}	Mg ha^{-1}	1 to 5	1 to 5	days
		Testcrosses across locations					
P65&66	4	1.92 ± 0.07	273.30 ± 43.4	2.28 ± 0.07	2.15 ± 0.07	2.85 ± 0.09	74.38 ± 0.20
P69	16	1.67 ± 0.03	199.35 ± 21.7	2.40 ± 0.03	2.23 ± 0.04	2.80 ± 0.04	74.18 ± 0.10
P70	7	2.07 ± 0.05	355.67 ± 32.8	2.09 ± 0.05	2.18 ± 0.05	3.18 ± 0.07	73.90 ± 0.15
P26	2	2.01 ± 0.09	438.29 ± 61.4	2.05 ± 0.09	2.60 ± 0.10	2.94 ± 0.12	74.53 ± 0.29
P33&34	5	1.96 ± 0.06	339.63 ± 38.8	2.22 ± 0.06	2.35 ± 0.06	2.95 ± 0.08	73.82 ± 0.18
Temp. x Trop. High-Oil	14	2.21 ± 0.04	496.14 ± 23.2	2.06 ± 0.04	3.36 ± 0.04	3.16 ± 0.05	73.61 ± 0.11
		Check Hybrids					
QPM	5	1.72 ± 0.06	230.60 ± 38.8	2.18 ± 0.06	2.02 ± 0.06	2.70 ± 0.08	75.24 ± 0.18
Opaque Crow's	3	2.17 ± 0.08	621.33 ± 50.1	2.56 ± 0.08	4.12 ± 0.08	3.09 ± 0.10	73.35 ± 0.24
Checks	4	1.93 ± 0.07	310.55 ± 43.4	3.19 ± 0.07	2.75 ± 0.07	2.53 ± 0.09	75.20 ± 0.20
		Means across locations					
All Hybrids	60	1.94 ± 0.02	342.53 ± 11.2	2.29 ± 0.02	2.62 ± 0.02	2.94 ± 0.02	74.12 ± 0.05
		Means at Single Location					
College Station, TX	60	1.1 ± 0.05	105.72 ± 13.1	2.2 ± 0.03	2.5 ± 0.04	2.8 ± 0.06	.
Weslaco, TX	60	2.1 ± 0.03	325.12 ± 24.0	3.0 ± 0.03	2.7 ± 0.02	3.1 ± 0.04	75.8 ± 0.11
Corpus Christi, TX	60	2.56 ± 0.02	596.78 ± 38.3	1.72 ± 0.03	.	.	72.4 ± 0.06
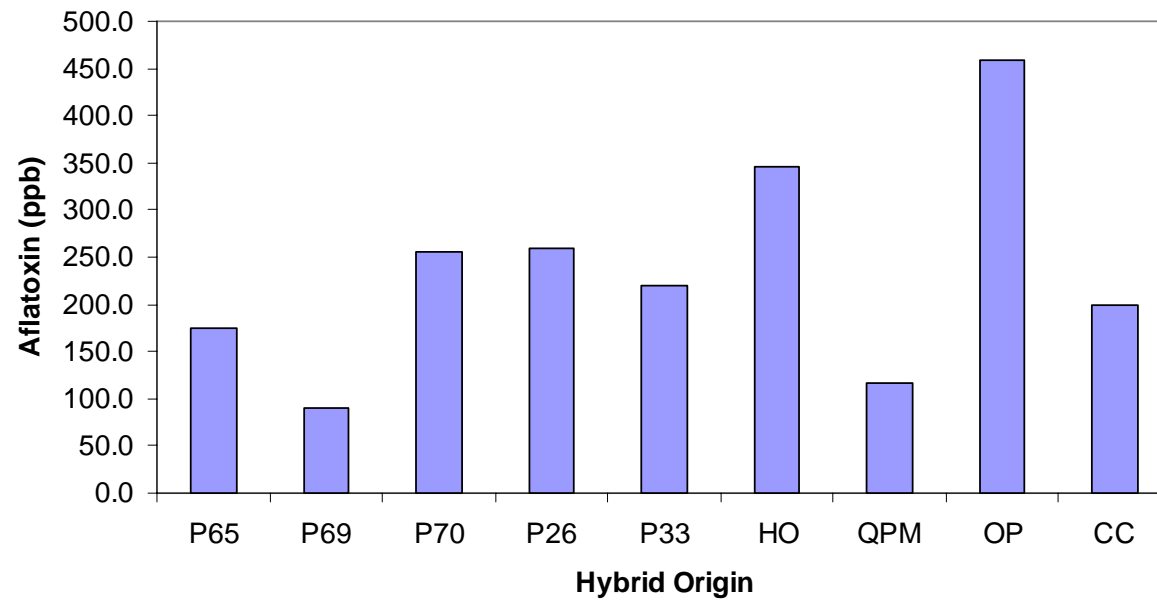
Fig. 3.1. Aflatoxin content for testcrosses of inbred lines with different origin across three Texas locations in 2002.

(Nielsen et al., 2002). Aflatoxin accumulation for standard non-opaque commercial hybrids was higher on average (310.5 ng g^{-1}) than for Population 69 (199.4 ng g^{-1}) and QPM hybrids (230.6 ng g^{-1}) (Table 3.5). These results suggest that there is enough variation among high lysine QPM inbreds to select for less susceptibility to aflatoxin than current commercial hybrids. Similar results were obtained with subtropical QPM hybrids by Bhatnagar et al. (2003) in evaluations at the same locations using the silk channel inoculation technique.

Average grain yields for testcrosses were 1.7 Mg ha^{-1} at CC, 3.0 Mg ha^{-1} at WE, and 2.2 Mg ha^{-1} at CS. Both the inbreds and their testcrosses showed significant variation for endosperm texture, kernel integrity, and maturities (Table 3.4 and 3.5). QPM inbreds and their testcrosses, except that inbreds from the Temperate x Tropical High-Oil population had flint endosperms, comparable to commercial hybrids and much harder than *opaque-2* commercial hybrids from Crow's. This indicates that it is feasible to develop high lysine lines with normal degree of endosperm modification. Inbreds from Population 69 and CML161 had the best rating for kernel integrity both in inbreds and testcrosses, whereas inbreds and hybrids with softer endosperm had the worst kernel integrity. We have observed a positive association between flint endosperm and better kernel integrity. Inbreds flowered in 80 days on average, 5 days later than their testcrosses (76 days) at WE. QPM hybrids had similar flowering dates than commercial hybrids (Table 3.5). These QPM inbreds are adapted to Southern US temperate environments and did not show photoperiod sensitivity in the testing environments.

Repeatabilities for aflatoxin and secondary traits in testcrosses and inbred lines

All the repeatibilities were significantly different from 0 (Table 3.6). Repeatibilities for aflatoxin ($R = 0.67$) and its logarithmic transformation ($R = 0.92$) in inbreds were relatively high compared with previous estimations (Betran et al., 2002) possibly due to higher amount of genetic variation present in this set of lines. Repeatibility estimates for aflatoxin accumulation in testcrosses measured across locations was 0.54 and showed a higher mean and range for aflatoxin accumulation at

Table 3.6. Family base repeatabilities and their standard errors for aflatoxin and associated traits per location and across locations in QPM inbred and their testcrosses.

Aflatoxin	Aflatoxin	Grain Yield	Endosperm Texture	Kernel Integrity	Silking date
log ng g^{-1}	ng g^{-1}	Mg ha^{-1}	1 to 5	1 to 5	days
		Repeatabilities for Inbreds at Weslaco, TX			
0.92 ± 0.11	0.67 ± 0.16	0.25 ± 0.41	0.65 ± 0.16	0.60 ± 0.26	0.67 ± 0.16
		Repeatabilities for Testcrosses at Single Locations			
		Weslaco, TX			
0.81 ± 0.04	0.62 ± 0.08	0.86 ± 0.03	0.95 ± 0.01	0.80 ± 0.04	0.62 ± 0.08
		College Station, TX			
0.45 ± 0.12	0.38 ± 0.14	0.64 ± 0.08	0.86 ± 0.03	0.48 ± 0.12	.
		Copus Christi, TX			
0.76 ± 0.05	0.66 ± 0.08	0.70 ± 0.07	.	.	0.54 ± 0.11
		Repeatabilities for Testcrosses at Across Locations			
0.72 ± 0.07	0.54 ± 0.10	0.87 ± 0.03	0.95 ± 0.01	0.66 ± 0.10	0.54 ± 0.16

WE (0.62), and CC (0.66) as compared to CS (0.38). Higher repeatability values observed at WE and CC could be due to more favorable environmental conditions prevailing at these environments for aflatoxin contamination in the testcrosses thereby increasing the amount of genetic variation among them. Greater genetic variation among the testing genotypes has been associated with greater repeatabilities (Falconer and Mackay, 1996; Betran et al., 2003b). Higher genetic variation among testcrosses could be due to lower frequency of favorable alleles in the tester (Smith, 1986). The tester Tx804 is an opaque soft endosperm inbred with open husks that has shown susceptibility in hybrid combination in previous evaluations, suggesting that the frequency of alleles for resistant factors to aflatoxin is low in Tx804. Another factor contributing to the genetic variance observed is that the lines used had an estimated coefficient of inbreeding greater than 0.97 after several generations of continuous selfing. The greater the inbreeding level of the testing inbreds, the greater is the genetic variation among

testcrosses (Hallauer and Miranda, 1988). Sufficient genetic variation was observed among these QPM lines with different origins for aflatoxin and associated traits suggesting that further selection among these inbreds can be effective.

Grain yield had the lowest repeatability in inbreds (R = 0.25). Grain yield repeatabilities in testcrosses were relatively high: 0.64 at CS, 0.86 at WE, 0.70 at CC, and 0.87 across locations. Endosperm texture had the highest repeatability at each location (0.86 at CS and 0.95 at WE) and across the two locations (R = 0.95). Endosperm texture is a trait that has shown consistent response across environments and high repeatabilities and heritabilities (Hallauer and Miranda, 1988). Visual rating for kernel integrity had a repeatability of 0.54 across locations. The causes of losing kernel integrity across locations can be different as the insect pressure and environmental conditions are different but in our study the ratings across replicates in one location and across the two locations were consistent enough to provide repeatabilities greater than 0.50. Silking date had a repeatability across locations of 0.54, which was relatively lower as compared with other reports (Hallauer and Miranda, 1988).

Correlations between secondary traits and aflatoxin contents

Significant phenotypic correlations were observed between aflatoxin and secondary traits for both inbreds and testcrosses across locations except for the correlation of grain yield and silking date with aflatoxin in inbreds (Table 3.7). In inbreds, aflatoxin was positively correlated with endosperm texture, kernel integrity, and grain yield, and negatively correlated with silking date. Less aflatoxin accumulations were associated with flinty endosperm texture, better kernel integrity, lower grain yield, and later maturities. In testcrosses across locations, aflatoxin was positively correlated with endosperm texture and kernel integrity, and negatively correlated with grain yield and silking date. The sign of correlations for inbreds and testcrosses was similar for all the traits except for grain yield. Less aflatoxin was associated with greater grain yields in testcrosses. Repeatabilities for grain yield in testcrosses were greater than

repeatability of grain yield in inbreds indicating more accurate estimates for association in the testcrosses. Similar results have been reported earlier (Betran et al., 2002).

Table 3.7. Phenotypic correlations for grain yield, grain texture, kernel integrity and silking date with aflatoxin content in inbreds at Weslaco and testcrosses across locations in 2002.

Trait	Units	Inbreds		Testcrosses	
		Aflatoxin	Aflatoxin	Aflatoxin	Aflatoxin
		$\log$ ng g^{-1}	ng g^{-1}	$\log$ ng g^{-1}	ng g^{-1}
Grain Yield	Mg ha^{-1}	0.40**	0.16	-0.32**	-0.30*
Endosperm Texture	1 to 5	0.53**	0.41**	0.49**	0.67**
Kernel Integrity	1 to 5	0.53**	0.37**	0.74**	0.60**
Silking date	days	-0.38**	-0.15	-0.35**	-0.50**

Both inbreds and testcrosses showed a similar trend for the correlations based on aflatoxin values and its logarithmic transformation suggesting that transformation did not change greatly the correlations (Table 3.7). The correlations among these traits reported here are valid only for this group of genotypes and environments. High correlations observed between aflatoxin and other agronomic traits such as endosperm texture, kernel integrity and silking date that have high repeatability could be useful in indirect selection for reducing aflatoxin contamination or combined in selection indices (Falconer and Mackay, 1996). Indirect selection can be more effective than direct selection when secondary traits show greater heritabilities (i.e., repeatabilities) than the primary trait and high correlations between secondary and target traits are present, or if greater selection intensities can be applied to the secondary trait. This is the case for traits such as endosperm texture and kernel integrity that are easy to screen in big populations and have high repeatabilities. The second approach would be to combine

information from several traits in selection indices (Baker, 1986; Lin, 1978). The incorporation of associated traits to aflatoxin contamination with high genotypic correlation with it such as endosperm texture, silking date, kernel integrity, husk coverage into the selection process can increase the rate of progress in developing less susceptible maize hybrids. Grain yield, stalk lodging and other agronomic characteristics can be added to the selection indices in order to select genotypes less susceptible to aflatoxin together with a desirable agronomic package that facilitates the deployment and use of sources of resistance.

Regressions of testcrosses on parental inbred lines

A total of 47 inbreds and their 47 corresponding testcrosses were used to estimate the relationship between inbreds and testcrosses through regression. Regressions of testcross values on inbred values were significant for all the traits. The slope was positive for all the traits except for grain yield (data not shown). Multiple R-values, equivalent to correlation between inbreds and testcrosses, were 0.78 for aflatoxin logarithmic transformation and 0.53 for aflatoxin (Fig. 3.2), and 0.20 for grain yield, 0.84 for endosperm texture, 0.48 for kernel integrity, and 0.60 for silking date (data not shown). These values indicate that except for grain yield the expression of endosperm texture, kernel integrity, silking date, and also aflatoxin in inbreds had predictive value for the expression of these traits in testcrosses. For this set of genotypes it seems plausible to select the less susceptible material based on the response of inbreds to aflatoxin contamination. Different results have been reported in other studies where the correlation between inbred and hybrids have been of low predictive value (Betran et al., 2002). The relationship between inbreds and testcrosses for associated traits is consistent with other studies that show that ear and kernel characteristics such as grain texture and kernel integrity have high correlation, while grain yield have low correlation (Hallauer and Miranda, 1988). Another important relationship is between the expression of
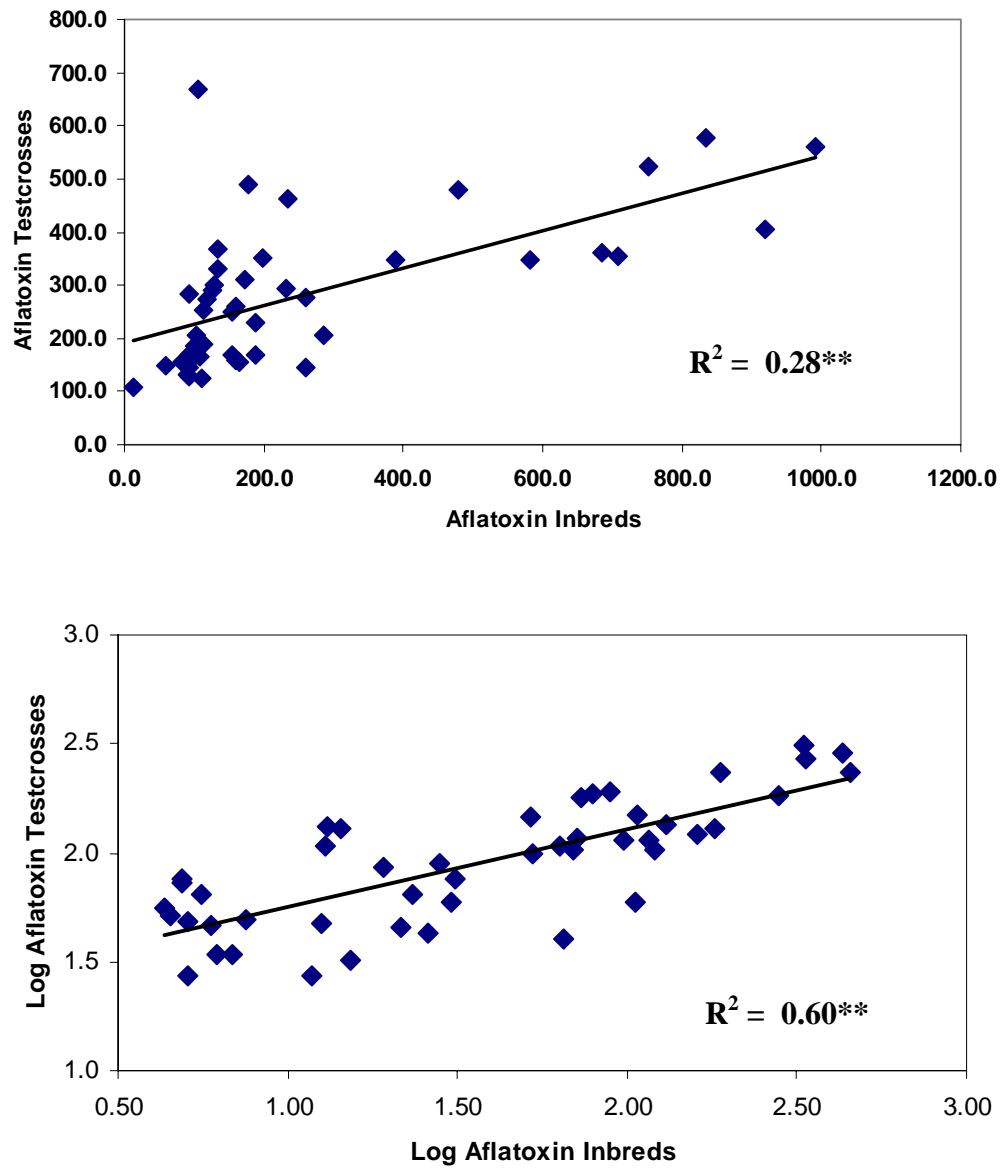
Fig. 3.2. Regression of aflatoxin and its logarithmic transformation across locations in testcrosses on means for inbreds at Weslaco, 2002.
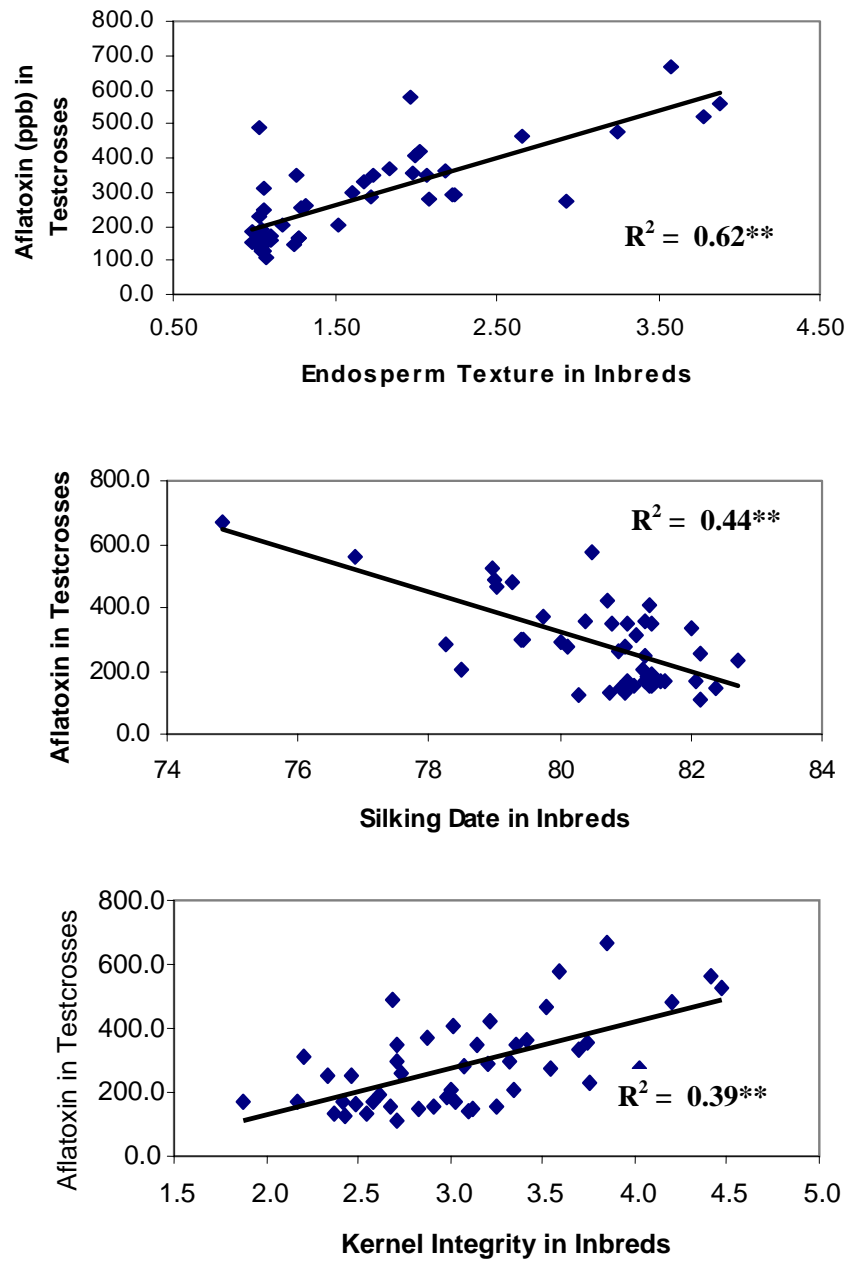
Fig. 3.3. Regression of aflatoxin across locations in testcrosses on means for associated traits in inbreds at Weslaco, 2002.

associated traits in inbreds and the response to aflatoxin of their testcrosses. Aflatoxin in testcrosses was significantly associated with endosperm texture (R^2=0.62), silking date (R^2=0.44), and kernel integrity (R^2=0.39) (Fig. 3.3). This suggests that for this material, selection for harder endosperm, late maturity, and good kernel integrity in parental inbreds can reduce the susceptibility to aflatoxin of their hybrids. The possibility to use inbred line information, as indicative of hybrid performance is desirable to reduce the number of hybrid evaluations. The predictive value of inbred line performance as indicative of hybrid performance can be variable depending on the environment, degree of inbreeding, lines and tester used, and the traits considered. Nevertheless, selection for additively inherited traits, such as maturity, endosperm texture, kernel integrity, and husk coverage would impact the response to aflatoxin contamination more than others (e.g., grain yield).

Quality evaluation

Relationship of tryptophan level with lysine content and endosperm modification

Tryptophan content in grain showed a significant and positive correlation with lysine content measured in all the inbreds and testcrosses (Fig. 3.4). These results indicate that in this set of germplasm measurement of tryptophan could be efficient in evaluating the amino acid quality of grain. Similar relationship between lysine and tryptophan has been reported earlier (Hernandez and Bates, 1969; Gentinetta et al., 1975). In all three sets of germplasm, tryptophan and endosperm modification showed a negative correlation (p<0.01) (Fig. 3.5). Wessel-Beaver et al., (1985) have reported similar negative correlations between tryptophan and endosperm modification. Furthermore, on grouping accessions within each set according to their level of endosperm modification as *o2o2* (endosperm modification < 3) or QPM (endosperm modification > 3), the mean tryptophan levels of the *o2o2* group was significantly (p > 0.05) greater than the QPM group in two of the three sets (Fig. 3.6). These observations are contrary to those reported by Pixley and Bjarnason, (2002) but similar to those

reported by Ortega and Bates (1983) and Robutti et al., (1974). Our study included a wide range of QPM, opaque and non-QPM germplasm, and consequently we had a greater range of endosperm modification values that helped us determine a better correlation. A negative correlation between tryptophan or lysine levels and endosperm modification could mean that selection for modified endosperm slightly reduces the beneficial effects of the *o2* mutation. This observation underscores the importance of monitoring amino acid levels throughout the breeding process, as has been suggested (Wessel-Beaver et al., 1985).
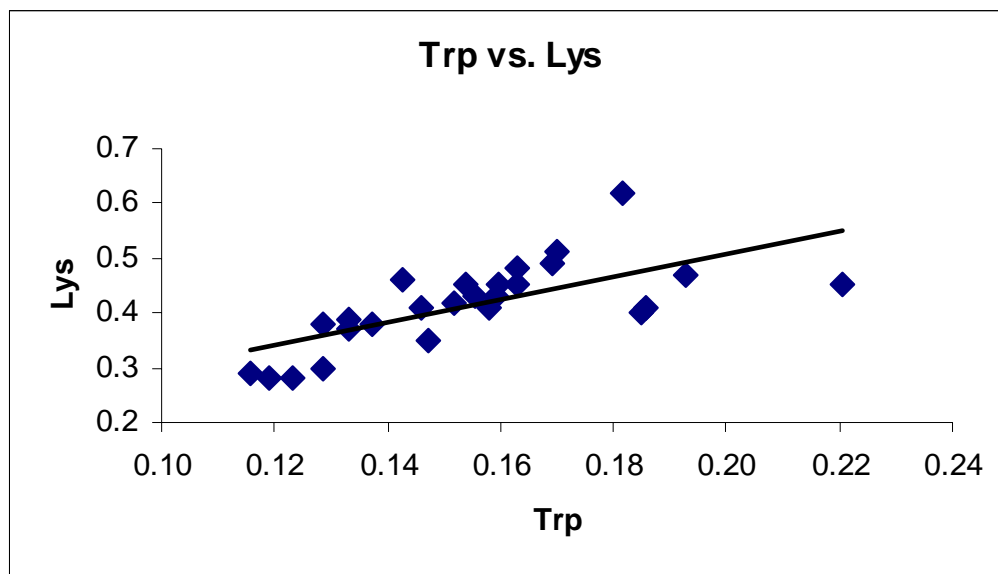
Fig. 3.4. Relationship between tryptophan (Trp, relative values) and lysine (Lys, mg/100 mg sample) for 28 maize genotypes (5 QPM hybrids, 6 QPM white inbreds, 10 QPM yellow inbreds, 4 normal inbreds and 1 normal hybrid).
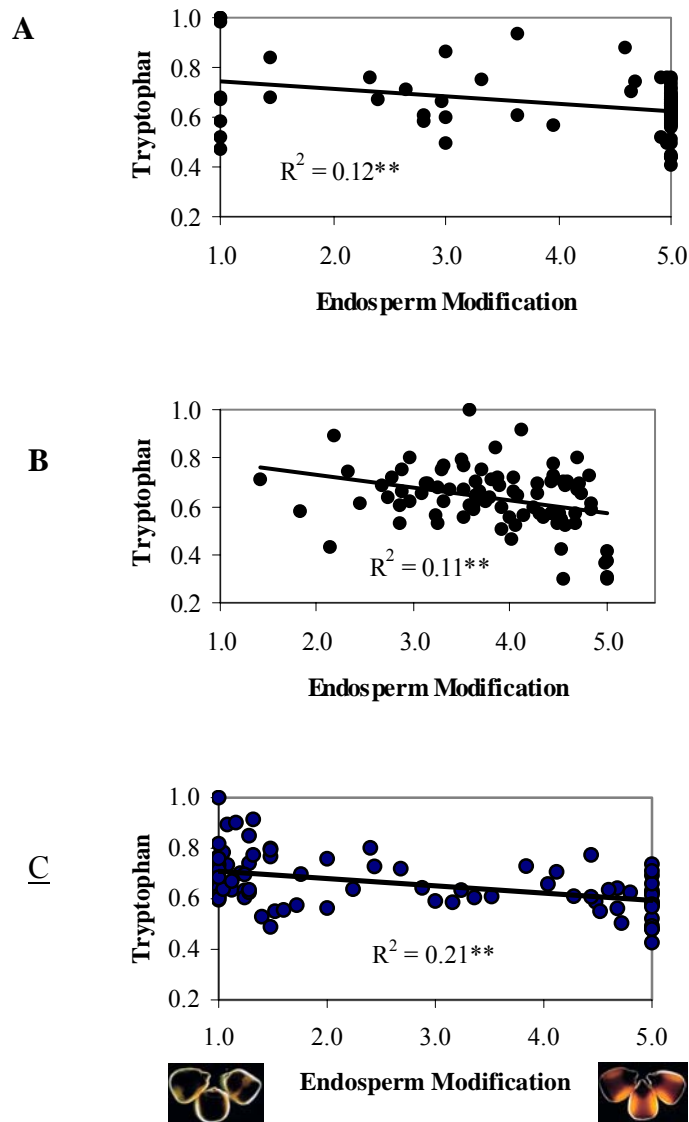
Fig. 3.5. Relationships between tryptophan and maize endosperm modification (1 = opaque, 5 = translucent) for inbreds 1 (A), testcrosses (B) and inbreds 2 (C).
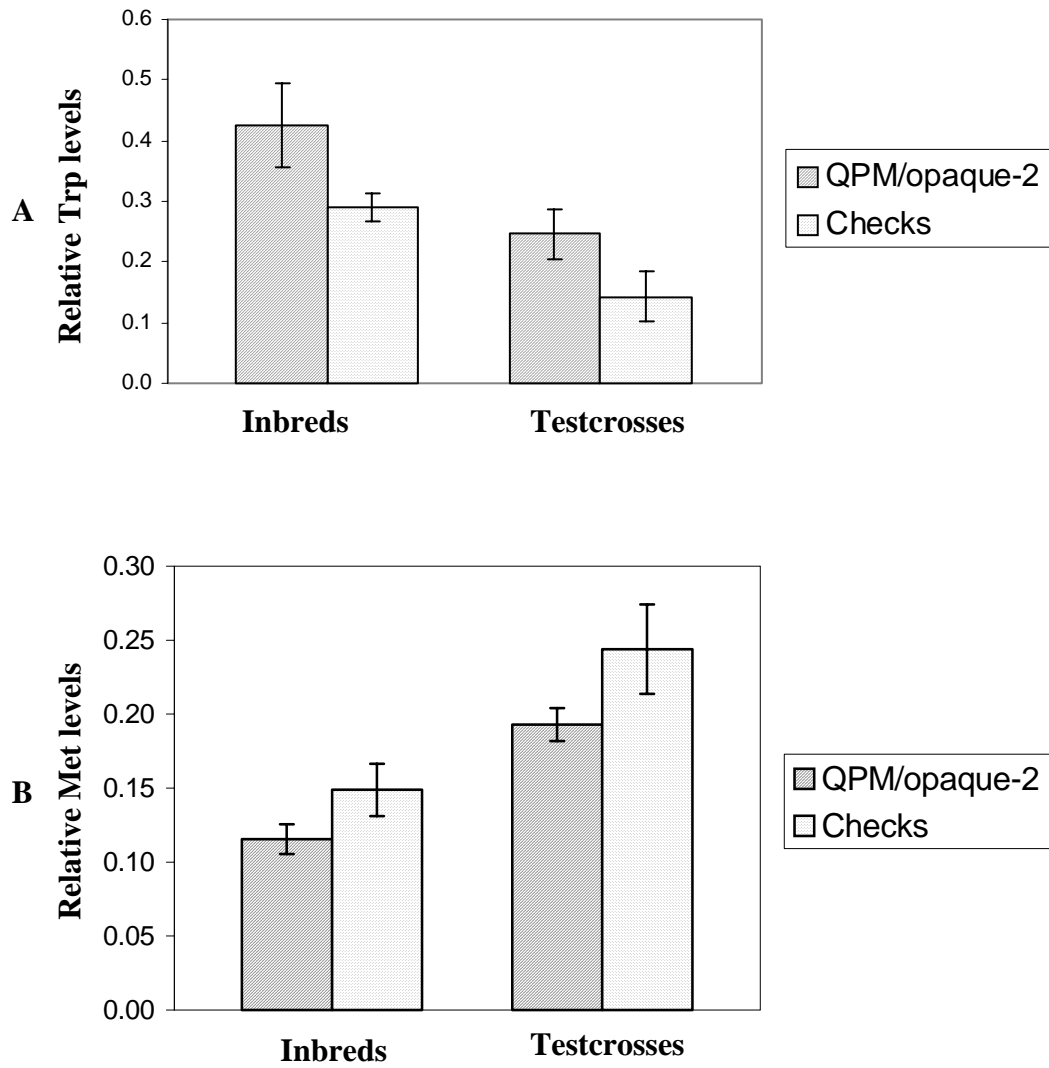
Fig. 3.6. Mean tryptophan (Trp) (A) and methionine (Met) (B) levels of QPM/opaque-2 and normal checks of inbreds (set 1) and testcrosses of QPM and opaque-2 inbreds with Tx804.
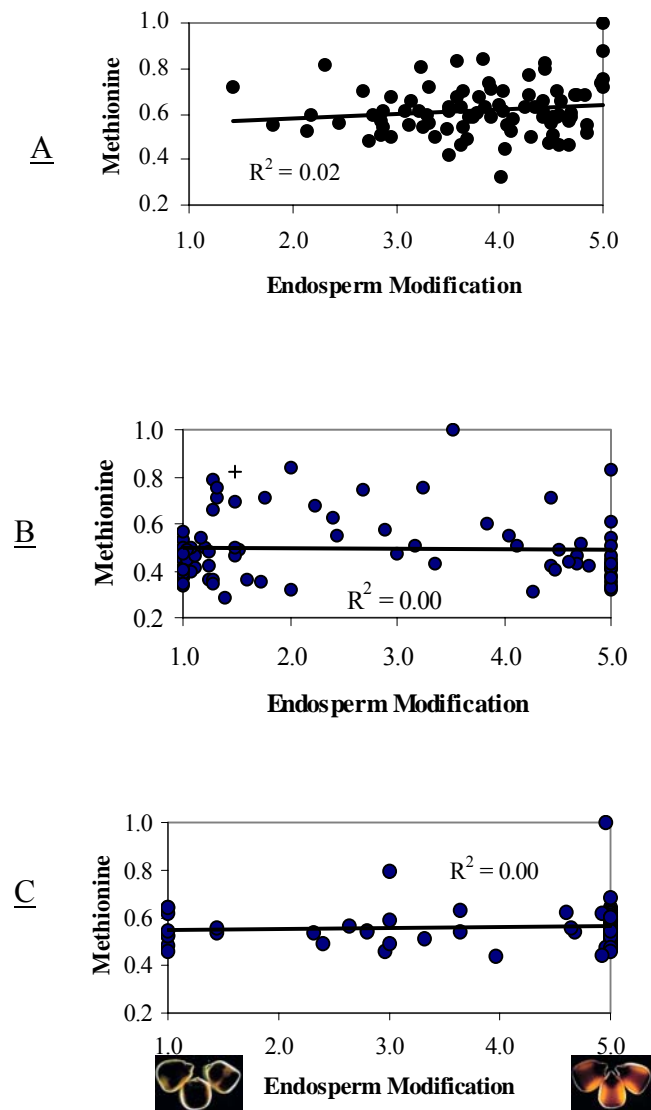
Fig. 3.7. Relationships between methionine and maize endosperm modification (1 = opaque, 5 = translucent) for inbreds 1 (A), testcrosses (B) and inbreds 2 (C).

Methionine evaluation in high lysine germplasm

      Methionine is the third limiting amino acid in maize used in non-ruminant diets after lysine and tryptophan, and it is the first limiting amino acid in the legumes. An analysis of methionine content in inbreds that were grouped as non QPM checks and QPM or opaque-2 (*o2/o2*) for each set of inbreds 1 and testcrosses (Fig. 3.6) revealed non QPM checks to be significantly higher in methionine levels as compared to the *o2o2* or QPM genotypes. Our results indicate that selection for modified endosperm does not significantly effect methionine levels, as was evident from the similar levels of methionine in both *o2o2* and QPM germplasm in all the three sets of germplasm evaluated (data not shown). Therefore, it may be possible to select simultaneously for both lysine and methionine levels to develop more nutritionally enhanced maize. Lastly no statistically significant relationship was observed between methionine levels and endosperm modification (Fig. 3.7).

Tryptophan and methionine levels in parental inbreds and their testcrosses

      The predictive values of tryptophan and methionine levels of inbred lines for tryptophan and methionine levels of testcross hybrid of each inbred with a common tester, Tx804, a NSS *o2/o2* tester with high lysine levels was examined. Regressions of tryptophan and methionine levels in 80 hybrids on *o2/o2* parental inbred lines with a range of endosperm modification were highly significant ($p > 0.01$) with R^2 values of 0.11 for tryptophan and 0.10 for methionine (Fig. 3.8). This indicates that the amino acid levels of parental inbreds have a low value for predictive amino acid levels in their hybrids. Most of the inbred parents (Inbreds 1) used in the regressions had vitreous endosperms (Fig. 3.5A and 3.7A), while Tx804 has soft endosperm. The testcrosses have a greater segregation of endosperm modification (F2 seeds between QPM x soft crosses) than their parents (Fig. 3.5B and 3.7B).
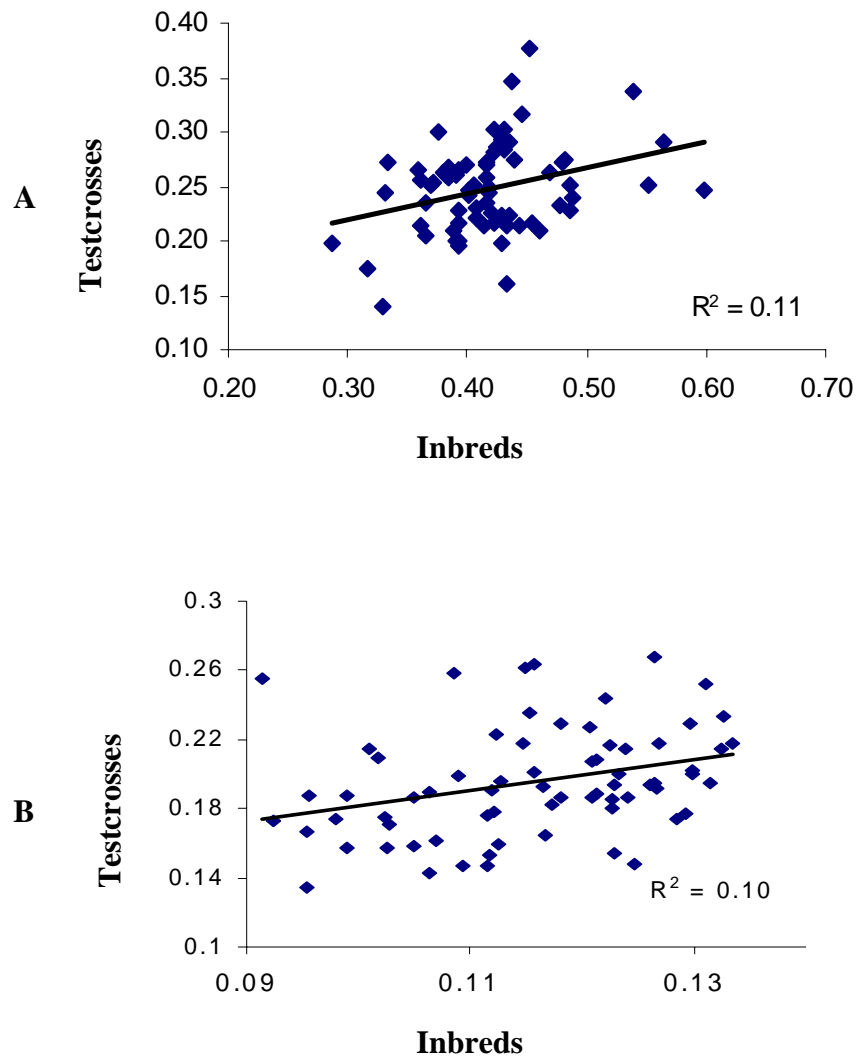
Fig. 3.8. Relationship between tryptophan contents (A) and methionine contents (B) in QPM inbred lines and their corresponding testcrosses with Tx804.
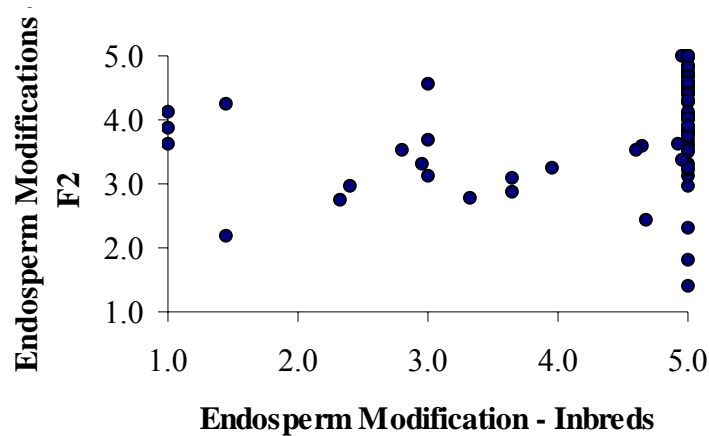
Fig. 3.9. Comparison of endosperm translucence between maize kernels from self-pollinated ears of parental inbreds and corresponding F2 kernels of their crosses with *o2/o2* inbred and Tx804.

Sixty-eight percent of the inbreds examined had fully vitreous kernels, but the F2 kernels derived from crosses of these inbreds with Tx804 gave a wider range of endosperm modifications scores (Fig. 3.9). There was no statistically significant correlation between the endosperm modification scores of the inbred lines with endosperm modification scores less than 5 and the F1 kernels derived from crosses of these inbreds with Tx804. We conclude that inbred line performance *per se* was not a good predictor of hybrid performance with a common *o2/o2* tester for tryptophan and methionine levels. However, it is feasible to evaluate different testers with variable levels of endosperm modification and amino acid levels and thereby develop a better understanding of the gene action controlling these traits. In QPM breeding programs, the majority of the effort is often devoted to altering the physical properties of the endosperm, maintaining the *o2* mutation, and improving agronomic traits. Our results indicate that it may be possible to further increase the nutritional value of QPM by selecting genotypes having both vitreous endosperm and high levels of these amino acids by careful monitoring the tryptophan and methionine levels during breeding.

CHAPTER IV

GENETIC DIVERSITY AND HAPLOTYPING OF MAIZE CHROMOSOME 7 IN *opaque-2* HIGH LYSINE INBREDS

OVERVIEW

Opaque-2 (*O2*) locus located on maize chromosome 7 regulates the transcription of 22-kDa alpha zein genes. Mutations at this gene (*o2*) increase lysine and tryptophan contents in maize endosperm. Our objectives were (i) to characterize haplotype variations along chromosome 7 of high lysine maize inbreds, particularly around the *opaque-2* locus (ii) to assess the level of genetic diversity of these inbreds in chromosome 7, (iii) to estimate the extent of linkage disequilibrium around the *opaque-2* locus and along chromosome 7, and (iv) to determine the parental contribution in some inbreds. Ninety-two inbreds with different origins [Stiff Stalk, Non Stiff Stalk, Pop 69 (CIMMYT), and combinations of temperate (Tx802, Tx804, Tx806, B97, B104) and exotic subtropical lines (CML 161 from CIMMYT and Do940y, Ko326y from South Africa)] were haplotyped on a cM scale utilizing 43 mapped SSR markers that were distributed uniformly along chromosome 7 but with more density of markers around the *O2* locus. In general, inbreds having common origin shared great proportion of similar haplotypes. Haplotypes around the *opaque-2* locus were similar between donor and converted lines. A total of 200 alleles were detected with an average of 4.7 alleles/locus (range 2 to 17). Dendrograms of genetic similarity estimates using the UPGMA method showed clusters in agreement with the different origin of inbreds. Significant linkage disequilibrium was detected around *opaque-2* locus spanning several cMs suggesting high selection pressure during the conversion of normal lines to *opaque-2*. Estimation of parental contribution identified haplotypes segments of chromosome 7 that were exclusively contributed by one or the other parent. These results can be useful in parental selection to create breeding populations that enhance genetic variation along

chromosome 7, and identification of parental inbreds that maximize heterozygosity in hybrid combinations.

INTRODUCTION

Maize (*Zea mays L.*) is a segmental allotetraploid that diverged more than 11 million years ago from two progenitor genomes (Gaut and Doebley, 1997; Gaut et al., 2000). The present maize genome came into existence through extensive segmental genome duplication that ranges from a few centimorgans (cM) to almost half of the chromosome (SanMiguel et al., 1998). The size of the maize genome is approximately 3 x 10^9 bp, which is roughly the same size as that of the human genome. However, genome size varies considerably by almost 50% within the species (Rayburn et al., 1985). It has been shown that inbred strains may differ considerably in transposon identity and copy number due to both hypervariable intergenic regions that are unrelated between inbreds, as well as highly variable genic regions (Fu and Dooner, 2002; Martienssen et al., 2004). Development of molecular markers has contributed extensively to the understanding of the complexities of the maize genome and facilitated the evaluation of genetic diversity in maize germplasm. Currently efforts are on to map the maize genome at several laboratories and a great deal of information about mapped markers (SSR's RFLPs and SNPs), which has been deposited at database (http://www.maizegdb.org) and is publicly available.

Genetic variation in the pattern and level of expression of gene products has been considered as one of the important factors contributing to diversity and adaptation of plants (Powell and Amato, 1984). Regulatory genes have been the focus of studies related to understanding of the process of diversification (Doebley, 1993). The *opaque-2* mutant gene *(o2o2)*, suppresses the transcription of the 22-kDa fraction (Burr and Burr, 1982; Pederson et al., 1982; Kodrzycki et al., 1989), which results in a decreased rate of transcription of the 22-kDa class of α-zeins. As a result there is an overall reduction in the proportion of α-zeins, a predominant fraction (60-75%) among the four major

classes of endosperm storage proteins, other fractions being albumins (3%), globulins (3%) and glutelins (34%). The zeins are almost devoid of essential amino acids lysine and trytophan, causing maize to be nutritionally inferior in protein quality as compared with rice, wheat and other major cereals. The reduced synthesis of α-zeins causes a concomitant increase in the proportion of other classes of storage proteins, albumins, globulins and glutelins, which are rich in lysine and hence better nutritional quality of maize with the mutant *opaque-2* gene. The *opaque-2* locus in maize has been shown to be highly polymorphic in both non-coding regions as well as protein-coding regions of the gene with evidence of high recombination rates and unequal distribution of polymorphism within the *opaque-2* sequence (Henry and Damerval, 1997).

The exploitation of *opaque-2* mutant gene in animal and human nutrition has recently gained importance and priority in the developing countries, where malnutrition in babies is a chronic problem (Vasal, 2001). Previous attempts to popularize high lysine maize carrying the *opaque-2* mutant gene were unsuccessful due to several agronomic deficiencies associated with the expression of *opaque-2* gene, such as lower grain yield, increased susceptibility to insects and pests in storage, poor kernel phenotype and lower kernel integrity (Vasal 2001). The International Center for Maize and Wheat Improvement, CIMMYT, developed Quality Protein Maize (QPM) that had higher yields, vitreous and harder endosperm and greater resistance to insects and pests by selecting simultaneously for modifiers conferring hard vitreous endosperm texture, higher tryptophan content and desirable agronomic properties. Currently a wide variety of subtropical and tropical populations, pools and hybrids with competitive yield and better quality are being used extensively in several countries in Asia, Africa and Central and South America. Texas A&M University (TAMU) has maintained a breeding program to develop QPM inbreds and hybrids with normal seed appearance, competitive yield and adaptation to Southern USA (Betrán et al., 2003 c,d,e). Selection has been conducted on diverse germplasm sources ranging from subtropical and tropical (from CIMMYT, México and University of Natal, South Africa) to temperate elite maize

inbreds that were previously converted to high lysine or were recycled to improve yield and adaptation to temperate growing conditions. The objectives of this experiment were:

(i)   to assess genetic diversity in chromosome 7 among 92 inbreds representing diverse origins using SSR markers

(ii)   to characterize haplotype variations along chromosome 7 of high lysine maize inbreds, particularly around the *opaque-2* locus,

(iii)   to estimate the extent of linkage disequilibrium around the *opaque-2* locus and along chromosome 7, and

(iv)   to determine the parental contribution in some inbreds.

REVIEW OF LITERATURE

Several maize mutants that affect protein quality have been identified over the past 30 years. A summary list of these mutants, their inheritance patterns, effect on zein accumulation, genetic map bin number, map coordinate position and source of map are presented in Table 4.1. Bins are sectors of genetic maps defined as "the interval of chromosomal segment that includes all loci from the topmost core marker to the next core marker (approximately 20 centimorgams apart) that define the bin boundary (locus or probe)" (Gardiner et al., 1993). Placement of a locus to a bin is dependent on the precision of mapping data and the resolution (the number of markers) of the map.

Table 4.1. Mutant genes, their inheritance patterns, effect on zein accumulation, bin no., coordinate position on map, and the source map type (Motto et al., 2003).

Locus	Inheritance	Effect on zein accumulation	Bin no., coordinate and source map
Opaque-1 (o1)	Recessive	unknown	4.07, 118, Genetic 4
Opaque-2(o2)	Recessive	22-kDa elimination 20-kDa reduction	7.1, 122.0 IBM

Table 4.1. Continued.

Locus	Inheritance	Effect on zein accumulation	Bin no., coordinate and source map
Opaque (os1)	Recessive	unknown	2.0, 43.1, Pioneer Composite 1999 2
Opaque-5(o5)	Recessive	Similar to *o1*	7.02, 58.10, Pioneer Composite 1999 7
Opaque-6(o6)	Recessive	General reduction	unknown
Opaque-7(o7)	Recessive	20-kDa reduction	unknown
Opaque-15(o15)	Recessive	27-kDa reduction Reduction in γ-zein	7.5, 136.6, Pioneer Composite 1999 7
Opaque-2 modifier1 (gzr1)	Semidominant	27-kDa overproduction	7.5, 152.0, Pioneer Composite 1999 7
Floury-2(fl2)	Semidominant	General reduction	4.0, 203.7, IBM neighbors v.2 4
Floury-3(fl3)	Semidominant	General reduction	unknown
*Defective Endosperm B30 (De*B30)*	Dominant	22-kDa reduction	7.01, 37.5, Pioneer Composite 1999 7
Mucronate (Mc1)	Dominant	General reduction	unknown
zp22	Recessive	22-kDa alpha zein gene cluster	4.02, 27.3, Pioneer Composite 1999 4
Zp27	Recessive	27-kDa zein protein cluster Regulated by *gzr1*. Identified by p-umc1216 (via SSR PCR)	7.02, 28.4, W64A/tester x tester 7
Zpr10(22)	Recessive	10-kDa reduction	4.02, 25.3, Pioneer Composite 1999 4
Ask1	Recessive	Threonine overproduction, regulated by O2	7.01, 44.0, Pioneer Composite 1999 7
Ask2	Recessive	Threonine overproduction, regulated by O2	2.06, 114.10, Pioneer Composite 1999 2

Hunter et al., (2002) characterized the protein and amino acid composition, and mRNA transcript profiles, of nearly isogenic inbred lines of W64A *o1, o2, o5, o9, o11,*

Mucuronate (*Mc*), *Defective endosperm B30* (*DeB30*), and *fl2* mutants in an effort to study the relationship between the phenotypes of these mutants and their biochemical bases. Their results indicated that the largest reductions in zein protein synthesis occur in the W64A *o2*, *DeB30*, and *fl2* mutants, which have almost 35 to 55% of the wild-type level of storage proteins. In the same study the pattern of gene expression in normal and mutant genotypes was assayed by profiling endosperm mRNA transcripts at 18 days after pollination with an Affymetrix GeneChip containing >1400 selected maize gene sequences. The results indicated increased expression of genes associated with physiological stress, and unfolded protein response, which were common features of the opaque mutants. All the mutants were classified into four major phenotypic groups based on their global patterns of gene expression *viz.* W64A+ and *o1*; *o2*; *o5/o9/o11*; and *Mc* and *fl2*. Studies conducted on *dek* mutants have reported nutritional, mechanical, and biotic stresses to also result in an opaque phenotype (Lyznik and Tsai, 1989; Neuffer et al., 1997).

The *Opaque-2* gene

To date about 18 mutants affecting endosperm protein storage synthesis in maize have been identified that alter zein synthesis and cause protein bodies with abnormal morphology, size, or number, and result in kernels with a soft, starchy texture. The first of these mutants identified was *opaque-2* (Mertz et al. 1964), a recessive gene located on short arm of chromosome 7 (bin no. 7.1, coordinate 122.0), which specifically eliminates the 22-kDa and reduces the 20-kDa α-zein fractions. The mutation *opaque-2 (o2)* results in small, unexpanded protein bodies (Geetha et al., 1991). The *Opaque-2* gene, a regulatory gene on chromosome 7, was isolated by transposon tagging and was found to encode a transcriptional activator of the basic leucine-zipper family of genes (Hartings et al., 1989; Schmidt et al., 1990). The O2 protein was shown to activate the transcription of the 22 kDa α-zein (Schmidt et al., 1992), the 14 kDa β-zein genes (Cord Neto et al., 1995), *b-32* (Lohmer et al., 1991), and *cyPpdk1* (one of two cytosolic isoforms of pyruvate orthophosphate dikinase) genes (Maddaloni et al., 1996). A comparison of wild

type *O2* allele and several variants of the recessive allele *o2* showed hypervariable regions in the N-terminal part of the gene with some variants (e.g., *o2-Crow*) showing large deletions in the coding region of the gene (lacking the zipper region and the C-terminal part of the gene) that results in a frame shift mutation and causes a premature termination of the polypeptide (Hartings et al., 1995). The defective or truncated opaque-2 protein results in significant reduction in the total zeins (almost eliminates the 22-kDa fraction and reduces the 20-kDa fraction) and consequently causing a pleiotropic increase in the amount of non-zeins proteins but not an increase in the lysine concentration of the non-zeins *per se* (Damerval and de Vienne, 1993; Habben et al., 1993; Moro et al., 1996).

Lohmer et al. 1991 showed that the *opaque-2* gene in developing endosperm also controls the expression of structural genes encoding an abundant albumin, termed b-32 and activates in vivo the promoter of the b-32 gene. Their studies also showed that the information necessary for this activation resides in a 440 bp DNA fragment containing five O2 binding sites (GATGAPyPuTGPu), of which two sites reside in copies of the 'endosperm box', a motif involved in endosperm-specific expression, which is also represented in 22 kd zein promoters. The O2 protein is also shown to be capable of binding in vitro and activating in vivo its own promoter.

Opaque-2 gene structure

The core of the *Opaque-2 (O2)* gene known as the *structural gene* consists of 1311 nucleotides that specify the composition of a transcription factor containing 437 amino acids (Schmidt et al., 1990). In the sequence published by Maddaloni et al. (1989), the nucleotide series is broken into six exons and five introns (Fig. 4.1). Both sequences published by Schmidt et al. (1990) and Maddaloni et al. (1989) differ in two main features: Maddaloni et al. (1989) characterized a sequence of 1548 nucleotides, whereas the upstream region of Schmidt et al. (1990) had only 258 nucleotides, and the length of the resulting protein sequence, which according to Schmidt et al. (1990) is a polypeptide with 437 amino acids, while Maddaloni et al. (1989) reported 453 amino acids.

Differences in the reported sequences may have occurred due to the methods used to isolate genes, the sequencing procedure, interpretation of the sequences and the genetic variation in the germplasm characterized.
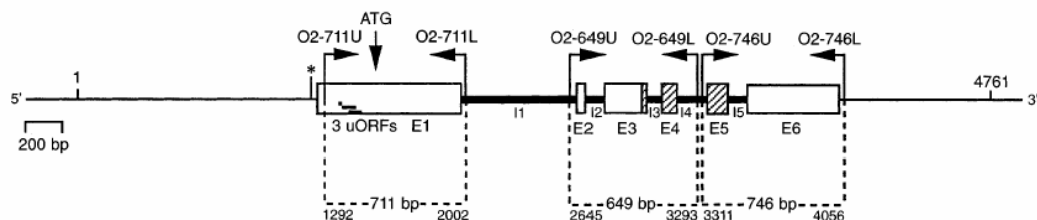
Fig. 4.1. Structure of the *Opaque-2* gene of maize. [Intron sequences are indicated by a horizontal heavy line; exon sequences are represented by boxes. The three heavy lines within exon 1 denote the three uORFs in the leader sequence. The start codon (ATG) is indicated and the asterisk represents the TATA box. Hatched boxes represent the region encoding the basic domain and leucine zipper of the transcription factor. Nucleotide positions are numbered according to the sequence published by Maddaloni et al. (1989)].

Opaque-2 endosperm modifiers

Quality Protein Maize (QPM) genotypes have been reported to contain increased amounts of cysteine rich 27-kDa gamma zeins (Ortega and Bates, 1983; Wallace et al., 1990; Lopes and Larkins, 1991) and have enhanced levels of mRNA (Geetha et al., 1991) that is regulated post-transcriptionally (Or et al., 1993). In a genetic analysis of F2 segregating seeds from crosses of *opaque-2* by QPM (modified *opaque-2*) genotypes, Lopes et al. (1995) indicated that the activity of *opaque*-2 is affected by the background of the parent. Their results also indicated that enhanced accumulation of gamma zeins in the endosperm are highly correlated with seed density (r^2 = 0.82), P < 0.01) and that degree of seed modification coupled with increased deposition of gamma-zeins were dosage dependent and directly correlated. Their results also indicated the presence of at least two complex modifier loci acting codominantly, one of which was mapped near the centromere of chromosome 7 and the other mapped near the telomere of chromosome

7L. The second locus called *gamma zein modifier1 (gzr1)* has been mapped in the Pioneer Composite 1999 7 Map at bin number 7.5 and coordinate 152.0 (http//:www.maizegdb.org). These two loci affecting endosperm modification, were associated with enhanced synthesis of gamma-zeins. This results in a vitreous phenotype caused by the formation of a larger number of protein bodies that creates an extensive proteinaceous matrix around the starch grains (Burnett and Larkins, 1999). However, further genetic analysis has revealed that although the gamma-zein locus may be necessary it is not sufficient to effect complete modification of the opaque-2 phenotype (Dannenhofer et al., 1995; Lopes et al., 1995; Lopes and Larkins, 1995). Recently a proteomic analysis of several QPM lines showed increased levels of granule-bound starch synthase I in the soluble nonzein protein fraction that was correlated with a change in starch structure, due to shorter amylopectin branches and increased starch-granule swelling. Gibbon et al. (2003) have reported alterations in starch structure associated with interconnections between starch granules to result in a vitreous kernel phenotype.

Yau et al. (1998) proposed the use of glutelins, which are the next major source of lysine in maize endosperm protein (4-5%), as an alternative to selection for high lysine maize. They reported significant correlation between lysine content in six out of seven glutelin proteins (35, 43, 48, 84, 92 and 100 k-Da). Glutelins are a multigene family composed of 3 subunits, G1-204 (28-kDa), G1-164 (16-kDa), G2 (15-kDa) and G3 (10-kDa). Complete cDNA sequences of the four genes amplified by PCR to determine the exact number of copies per genome showed that G1 has 5-10 copies, G2 has 1-2 copies and G3 has 2-3 copies per genome (Lazzari et al., 1993). The structure and amino acid sequence of the *glutelin-2* gene has been determined (Prat et al., 1985) and isolation and sequencing of a 28-kDa *glutelin-2* gene has been done (Boronat et al., 1986).

Other major genes identified in maize chromosome 7 and their main characteristics

Opaque endosperm15 (**o15**) **–** It causes small opaque poorly viable kernel, abnormal seedling and plant (dwarf, ear in tassel) and reduces the 27-kDa gamma zein mRNA and protein. The *opaque-15* mutation maps near the telomere of chromosome 7L, coincident with an *opaque-2* modifier locus and appears to be a mutation of the *opaque-2* modifier gene (*gzr1*). This locus has been mapped at bin no. 7.5, coordinate 136.6 in the Pioneer Composite map 1999 7. The *o15* mutant that reduces γ-zein synthesis leads to a smaller number of protein bodies (Dannenhoffer et al., 1995). In QPM there is overproduction of γ-zein that enhances protein body number and result in the formation of more vitreous endosperm (Lopes et al., 1995; Moro et al., 1995).

Defective Endosperm B30 (De*B30) – It is a mutant dominant allele that causes opaque, high lysine endosperm (Balconi et al., 1998) that has been mapped in the Pioneer Composite 1999 7 map at bin number 7.01 and coordinate 37.5. The *Defective endosperm B30* (*DeB30*), along with other opaque mutants, such as *floury2* (*fl2*) and *Mucuronate* (*Mc*), are associated with irregularly shaped protein bodies (Fontes et al., 1991; Coleman et al., 1997c).

Opaque endosperm5 (o5) **–** It causes opaque kernel, light yellow endosperm and sometimes modified to shrunken-like sh1 or sugary-like su1. Seedlings are virescent to yellow or white, depending on allele with some being lethal. This locus has been mapped in the Pioneer Composite 1999 7 map at bin number 7.02 and coordinate 58.10.

Opaque endosperm (o1) - The *o1* mutation has little effect on zein synthesis (Nelson et al., 1965), yet results in a soft, starchy endosperm. This mutation has been mapped at bin no. 4.07 and coordinate 118 on Genetic 4 map.

Locus 27-kDa zein protein (zp27) **–** This mutant allele has been mapped on the short arm of chromosome 7 (coordinate 28.40 and bin number 7.02) in the population W64A/tester x tester 7 (Esen, 1982).

Lysine biochemical and catabolism pathway

The aspartate pathway has been studied in detail that leads to increase in lysine production (Azevedo et al., 1997). The first regulatory enzyme in the pathway is aspartate kinase (AK) that leads to the biosynthesis of lysine, methionine, threonine and isoleucine. The *Ask1* gene encodes an AK isoenzyme sensitive to lysine inhibition and is regulated by O2 causing alterations in the level of soluble amino acids, total amino acids, storage proteins and enzyme activity (Azevedo et al., 1990; Brennecke et al., 1996). Genetic analysis showed that both genes are linked on chromosome 7 (Azevedo et al., 1990). Another gene *Ask2* that encodes an aspartate kinase and is also sensitive to lysine inhibition could be a QTL for free amino acid content in *o2* mutants (Wang and Larkins, 2001; Wang et al., 2001).

Lysine catabolism via the saccharopine pathway is the major route for lysine degradation in plants (Azevedo and Lea, 2001). Studies on investigation of activity of LKR (lysine-ketoglutarate reductase) and SDH (saccharopine dehydrogenase), the first two enzymes in the pathway of lysine degradation via aminoadipic acid, respectively, in homozygous normal and opaque-2 versions of two inbreds (ML649 and L438) revealed LKR activity of L438 opaque-2 endosperm to be three times lower than the activity for L438 normal and almost no enzyme activity in the ML649 opaque-2 inbred line, whereas there was no change in the activity of SDH due to *opaque-2* gene (Arruda et al., 2000). They concluded that the decrease in LKR activity of opaque-2 endosperm resulted in decrease in rate of lysine degradation and was not due to the presence of an enzyme inhibitor in the mutant endosperm. In another study, it was shown that the first two enzymatic steps are catalyzed by lysine-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) as two parts of a bifunctional polypeptide (LOR/SDH) and the LOR activity was decreased by a factor of 2 to 3 in *o2* mutants as compared to wild-types (Brochetto-Braga et al., 1992). Sequencing the cDNA corresponding to LOR/SDH revealed that the SDH activity was encoded by the C-terminal part of the messenger, while the N-terminal sequence encoded the LOR enzyme (Kemper et al., 1999). The genomic sequence of the gene and its 5' regulatory regions

have revealed the presence of O2 boxes in the upstream promoter (Arruda et al., 2000), that cause transcriptional control of the *Lor/Sdh* gene by *O2*. In a study of activities of enzymes of the aspartate pathway and lysine catabolism in two QPM, an *o2* mutant, and a wild-type *O2* varieties, higher AK activity and a lower LOR and SDH activity was observed in the QPM as compared to the wild-type and mutant *o2* genotypes. This suggests the role of modifier genes in enhancing the effects of o2o2 on the LOR/SDH (Gaziola et al., 1999). Studies conducted on the association of other genes having effect on non-zein proteins showed EF 1-α, a lysine rich protein (10%) which binds the aminoacyl-tRNAs to the ribosome to be directly and significantly correlated (r^2= 0.9) to total lysine content of the endosperm protein (Larkins et al., 1996; Habben et al., 1995).

A list of maize proteins that are affected by *opaque-2 (o*2) mutation, their function and type of regulation is presented in Table 4.2.

Table 4.2. Maize proteins that are affected by *opaque-2 (o*2) mutation, their function and type of regulation (Motto et al. 2003).

Protein	Function	Up/down regulation [a]	Transcriptional Regulation
22-kDa zein	Storage protein	Down	Yes
b-32	TypeI ribosome-inactivating protein	Down	Yes
b-70	HSC70 homologue	Down	No
Cytosolic PPDK	Amino acid interconversions	Down	Yes (unknown)
Aspartate aminotransferase	Amino acid interconversions	Down	unknown
Acetohydroxy-acid synthase	Synthesis of branched chain amino acids	Down	unknown
Glyceraldehyde 3P-dehydrogenase	Stress-induced glycolysis	Up	unknown
Aspartic proteinase precursor	Proteolysis during germination	Up	unknown

Table 4.2. Continued.

Protein	Function	Up/down regulation [a]	Transcriptional Regulation
Lysine ketoglutarase reductase	Lysine catabolism	Down	unknown
Aspartate kinase	Lys, Met, Thr synthesis	Down	unknown
Elongation factor 1 alpha (EF 1-α)	Translation, mRNA-cytoskeleton association	Up	unknown

[a] Changes relative to normal

Genetic diversity in maize

Genetic diversity is a measure of variability among individuals determined by a specific method or a combination of methods (Mohammadi and Prasanna, 2003). Understanding genetic relationships and diversity, at different levels such as germplasm pools and populations or inbreds in maize is critical to maximize efficiency in any maize breeding program. Recent applications of molecular markers in the assessment of genetic diversity have facilitated diverse studies in maize and have been valuable in diverse applications for crop improvement strategies (Betran et al., 2003a). Genetic diversity can be estimated in inbred lines (homozygous genotypes) and populations (mixture of genotypes with variable degree of heterozygosity). The genetic diversity present in both inbreds and populations is affected by several genetic phenomena such as linkage, migration, inbreeding and population substructure. Genetic diversity measures in populations are dependent on several factors such as number of individuals sampled, number of loci, mating system, genotypic and allelic frequencies, and the effective population size (Weir, 1996b).

Genetic diversity has been studied in detail using diverse data sets in the past, eg. pedigree data (Bernardo, 1993), morphological data (Smith and Smith, 1992), isozyme data (Hamrick and Godt, 1997), and recently DNA based (RFLP's, AFLP's, SSR's SNP's) data (Bhattramakki and Rafalski, 2001). Diversity studies using pedigree data

have utilized Malecot's (1948) coefficient of coancestry (f), which is the probability of two random alleles sampled from each of two individuals to be identical in descent. However, this measure fails to take into account common violations of assumptions in plant breeding programs such as genetic drift due to small population size, selection and ambiguity in pedigree records (Messmer, et al., 1993). Variation in isozymes has been used to estimate genetic distance in 31 maize lines that was consistent with pedigree data (Stuber and Goodman, 1983), however the discriminatory power of isozymes as markers is limited mainly due to few polymorphic loci reported in plants. The development and use of molecular markers has facilitated greatly the assessment of genetic diversity at the DNA level (Melchinger and Gumber, 1998). Genetic divergence studies utilizing RFLP's as DNA markers have been reported to provide accurate measures of genetic distance that are consistent with pedigree information (Lee et al., 1989 and Melchinger, 1990) and analyzing relationship between genetic distance and heterosis (Stuber, 1989; Smith et al., 1990; Boppenmeier et al., 1992; Dudley, 1994; Dubreuil et al., 1996), however, their effective utilization is limited by the labor intensive and time consuming nature of RFLP analyses. Studies conducted at Texas A&M by Kata et al. (1994) reported an effective and accurate marker assay method for identifying plants with O2/O2, O2/o2 and o2/o2 genotypes from juvenile leaf DNA samples by using *Opaque-2* cDNA developed from RFLPs as a probe on HindIII-digested genomic DNA.

Use of single sequence repeats (SSR's) or microsatellites in maize

Microsatellites or single sequence repeats (SSRs) are short (2 to 6 nucleotides) tandemly arranged DNA sequences. Microsatellites, PCR based codominant markers abundant in several species including maize (Powell et al., 1996), are highly polymorphic (Smith et al., 1997; Beckmann and Soller, 1990; Senior and Heun, 1993; Matsuoka et al., 2002), easy to analyze by automated systems (Sharon et al., 1997), highly accurate and repeatable (Heckenberger et al., 2002), and have been mapped extensively over the entire maize genome (Chin et al., 1996; Taramino and Tingey, 1996). In diversity studies utilizing mapped SSR's is advantageous in uniform and

controlled sampling of the genome, which is important in avoiding possible overrepresentation of the genome leading to inaccurate estimation of genetic distances (Menz et al., 2004). Further the efficiency of SSR based genetic analyses can be increased by multiplexing reactions using automatic electrophoretic conditions (Mitchell et al., 1997). In a comparative study of different types of markers (AFLPs, RAPDs, SSRs, and RFLPs) in classifying 33 inbred lines, SSRs identified twice the number of alleles than AFLPs and RAPDs, and were 40% more informative than RFLPs (Pejic et al., 1998).

Analyzing molecular diversity at any level has been useful in understanding past selection history, genetic drift, recombination and populations structures in maize germplasm. Inbreds in maize have been a valuable resource in diversity studies, such as development of linkage maps (Burr et al. 1988), quantitative loci mapping (Edwards et al., 1987; Austin et al. 2001), molecular evolution (Henry and Damerval, 1997; Ching et al., 2002), developmental genetics (Poethig, 1988; Fowler and Freeling, 1996), classification into heterotic groups (Stuber, 1989; Lee et al., 1989; Boppenmeir et al., 1992; Melchinger, 1993; Betran et al., 2003a) and in evaluating linkage disequilibrium and association genetics in maize (Remington et al., 2001; Thornsberry et al., 2001).

Several studies have focused on analyzing the genetic diversity in maize inbred lines developed in breeding programs around the world utilizing molecular markers. Commonly used temperate inbreds in both public and private breeding programs in the USA have been classified on the basis of molecular markers into two major heterotic groups (Iowa Stiff Stalk Synthetic (BSSS), and Non-BSSS) and a third group of unrelated lines to either group (Mumm and Dudley, 1994; Dubreuil et al., 1996; Dubreuil and Charcosset, 1999; Gethi et al., 2002). Liu et al. (2003) estimated the genetic structure and diversity in an analysis of 260 maize inbred lines, including many of the known publicly available temperate, tropical and subtropical maize lines using 94 SSRs. Five major groups were identified representative of major breeding groups (temperate Stiff Stalk, temperate Non Stiff Stalk, tropical and subtropical germplasm,

popcorn and sweet corn) with greater number of alleles identified in the tropical and subtropical germplasm in comparison to temperate germplasm.

Researchers at International Center for Maize and Wheat Improvement (CIMMYT), Mexico have utilized molecular markers extensively to characterize subtropical and tropical germplasm and assigning breeding lines and populations into heterotic groups using DNA finger printing techniques (Warburton et al., 2002; Reif et al., 2003; Reif et al., 2004). DNA finger printing is a powerful technique for assessing genetic diversity at the DNA level in plants (Melchinger and Gumber, 1998) and genetic distances based on molecular marker data in conjunction with phenotypic data is extremely useful in classifying heterotic groups and identifying promising heterotic patterns among maize genotypes (Melchinger, 1999). Genetic characterization of Asian maize inbred lines utilizing molecular markers classified the inbreds developed in China and Indonesia into 6 majors groups with lines developed from CIMMYT germplasm falling into separate category (George et al., 2004).

Molecular markers, especially SSR's owing to their high reproducibility have been also utilized in estimating genetic relationships between inbreds and the extent of haplotype sharing within diverse groups of maize inbreds when pedigree data is available (Romero-Severson et al., 2001).

Statistical methods and software's to analyze genetic diversity

Several commonly used measures to analyze genetic diversity include:

Genetic distance (GD) – It is defined as "any quantitative measure of genetic difference, at the sequence level, allele frequency level, that is calculated between individuals, populations or species" (Beaumont et al., 1998). Genetic distance has been used extensively in plant breeding to group inbreds, populations or cultivars. Genetic similarity (GS), the opposite of GD (GS = 1-GD), is commonly used in clustering plant inbreds, populations and cultivars utilizing molecular markers (SSRs, AFLPs, RFLPs, SNPs). A binary matrix of 1 (presence of allele) and 0 (absence of allele) is used in several statistical methods such as Nei and Li's (1979) coefficient, Jaccard's coefficient (1908), simple matching coefficient (Sokal and Michener, 1958), and Modified Roger's

distance to estimate GD. Mohammadi and Prasanna (2003) have reviewed the commonly used methods employed in analyzing genetic diversity.

Multivariate methods – Several methods used in analyzing genetic diversity and classifying plant populations, inbreds and cultivars are:

(1) Cluster analysis that are either distance based methods (further classified as hierarchical or nonhierarchical) or model based methods (maximum-likelihood based or Bayesian based methods). Most commonly used methods used in hierarchical clustering are UPGMA (Unweighted Paired Group Method using Arithmetic averages) (Sneath and Sokal, 1973) and Ward's minimum variance method (Ward, 1963).

(2) Ordination methods such as principal component analysis and principal coordinate analysis are methods of data reduction by linear transformation of the original variables into a set of new uncorrelated variables known as principal components (PCs) (Johnson and Wichern, 1992). Generally one or more PCs are used to generate 2 or 3-dimensional scatter plots to establish the genetic distance between individuals.

(3) Multidimensional scaling methods in which molecular marker data can be conveniently viewed in 2 or 3-dimensions using similarity or distance matrix (Schiffman et al., 1981; Beebe et al., 1995).

Several software's are currently available to analyze molecular marker data analysis in diversity studies with applications in estimating common diversity measures, infer population structure, perform clustering, test for Hardy-Weinberg and multilocus equilibrium and calculating other population statistics. Commonly used programs are NTSYS, TFPGA, FSTAT, PHYLIP Arlequin, GDA, Genepop, Genestrut, Popgene, Structure, Tassel, Powermarker etc. (Appendix D) for analyzing population structure, phylogenetic analysis and performing association analysis.

Linkage disequilibrium (LD) and association studies in maize

Linkage disequilibrium is defined as the "non random association of alleles at different loci". High LD exists whenever there is linkage or the population is subject to selection, genetic drift or admixture. Linkage disequilibrium is greatly influenced by

several factors such as population structure, recombination hot spots and mating system. Typically, LD mapping can provide a resolution of 1-2 centimorgan around the gene of interest, however, it can be increased by population-based studies (Devlin and Risch, 1995). Several measures to study LD have been used (Hedrick, 1987 and Jorde, 2000), however the two most common measures used are D' (Lewontin, 1988) and r^2 (Hill and Weir, 1994). Lewontin's D' is calculated as:

$|D'| = (D_{ab})^2/min(p_Ap_b,p_ap_B)$ for $D_{ab} < 0$;

$|D'| = (D_{ab})^2/min(p_Ap_B,p_ap_b)$ for $D_{ab} > 0$

The square of the standardized measure (r^2) is calculated as:

$r^2 = D_{ab}{}^2/(p_A+p_B+p_a+p_b)$

In both the measures D_{ab} is calculated as

$D_{ab} = (p_{AB} - p_Ap_B)$

In association studies commonly r^2 values are used as D' values are strongly affected by small population sizes giving biased values when comparing alleles with low frequencies. Linkage disequilibrium studies have been used extensively in humans to identify genetic regions associated with a particular disease, eg. cystic fibrosis gene (Kerem et al., 1989). Several studies have analyzed LD and its patterns in plants particularly in Arabidopsis (Nordborg et al., 2002)

In maize several studies have investigated patterns of LD over a wide range of populations and marker types ranging from SNPs within sequenced genes to SSRs across the genome. Remington et al. (2001) in a survey of six candidate genes using SNPs reported rapid decline of intragenic LD ($r^2 < 0.1$ within 1500 bp) indicating large effective population sizes in maize during evolution and high levels of recombination. In the same study they used 47 SSR markers among 102 diverse inbred lines and reported a strong evidence of genome-wide LD that could be reduced by grouping the lines into three empirically determined subpopulations. Palaisa et al. (2003), in the study of effects of selection on sequence diversity and LD at two phytoene synthase loci (*Y1 and PSY2*),

reported rapid decline in pairwise LD measured as r^2 (0.1 within 1000 bp) in white endosperm lines for the *Y1* locus, whereas in the yellow endosperm observed r^2 levels decreased rapidly within 250 bp. Similar observations have been reported by Tenaillon et al. (2001) in a study of sequence diversity in 21 loci distributed along chromosome 1 of maize. In their study LD (measured as expected value of r^2) decayed rapidly over a short distance on average (0.15 within 500 bp).

However, Rafalski (2002) found contrasting evidence of persistent LD in regions of DNA greater than 100 kb for *adh1* and *y1* loci in elite maize populations and in a similar study found no decay in LD in 300-500 bp range. These differences in LD decay could be possible due to several reasons including population history and low rates of recombination in repetitive regions of the maize chromosome (Flint-Garcia et al., 2003). Ching et al. (2002) in a study of the frequency and distribution of DNA polymorphisms at 18 maize genes in 36 maize inbreds, that represented most of the genetic diversity in U.S. elite maize breeding pool reported no rapid decline of linkage disequilibrium within a few hundred base pairs in the elite maize germplasm, which was consistent with the effects of breeding-induced bottlenecks and selection on elite germplasm pool. Their results indicated large genetic distance between haplotypes, which is indicative of an ancient gene pool and of possible interspecific hybridization events in maize ancestry.

Estimation of parental contribution in breeding lines

Parental contribution defined as the "proportion of the genome derived from the recombinant inbred from its parental inbreds" is a useful method in determining the genetic relatedness between inbreds utilizing molecular markers (Bernardo, 2002). A comparison of molecular (RFLP, SSR) and pedigree data (coefficient of coancestry) was done to evaluate parental contribution of inbreds to their progeny (Bernardo et al., (2000). Their results revealed significant differences between the molecular and pedigree data estimates but no significant differences were observed between the two molecular marker estimates. In both molecular estimates the sum of the parental contributions did not equal 1.0 due to non-parental bands, which could be due to several factors such as:

residual heterozygosity, contamination, mutation or recombination within a band. RFLP bands showed more non parental bands than SSR markers.

Romero-Severson et al. (2001) utilized genetic similarities and haplotype sharing within a diverse set of inbreds from North America and Europe including Iodent, Iowa Stiff Stalk Synthetics (BSSS) Lancaster Surecrop, flint types, sweet corn and popcorn. The iodents showed shared haplotypes on several chromosomes (1,3,4 and 10). The B73 group, popcorns and flint corns showed extensive haplotype sharing on chromosome 9 with a high proportion in a 20 cM region on chromosomes 3 and 10.

MATERIALS AND METHODS

Plant material

Ninety high lysine maize inbreds developed at the Maize Breeding and Genetics Program, Texas A&M and two inbreds developed at CIMMYT, Mexico (CML 161) and University of Natal, South Africa (Do940y) were included in this study (Appendix D, Table D.1). These inbreds representing a wide range of genetic backgrounds have different origins: Temperate Stiff Stalk and Non Stiff Stalk germplasm, CIMMYT QPM Populations 65, 69, 70 and temperate x tropical high oil, temperate inbreds (Tx802, Tx804, T220o2, B73o2), tropical exotic inbreds CML 161 and Do940y, and inbreds derived from crosses between temperate and exotic germplasm.

Phenotypic data for endosperm modification was estimated using a weighted average following a 1 to 5 scale (opaque = 1, semi-vitreous = 3, and vitreous translucent = 5). Genotypes with *o2/o2* grain with a score greater than 3 were defined as QPM.

DNA extraction

Ten seeds of each inbred were germinated in 90 mm petri dishes on filter papers saturated with a weak fungicide solution (captan) and incubating them in dark at 28^0C for 1 week. A 50 mg sample of freshly emerging coleoptiles were harvested and bulked in 1.5 ml microcentrifuge tubes and DNA was extracted from this tissue following the DNAzol protocol as described below with modifications described by Emani et al.

(unpublished). Tissues were suspended in 350μl sucrose buffer (11.89% sucrose, 100mM Tris-HCl [pH 8.0], 5 mM EDTA[ph 8.0]), 7 μl β-mercaptoethanol and 7 μl of 10 mg/ml RNAase A solution. Tissues were ground in Genogrinder for 30 seconds after which 350 μl of Plant DNAzol reagent (Invitrogen) was added and the mixture was gently inverted for 10 min. The suspensions were extracted with an equal volume of chloroform and supernatant was collected by centrifugation. DNA was precipitated with 0.75 vol. absolute ethanol and pelleted by centrifugation. The pellets were sequentially washed for 5 min each with 150 μl of DNAzol-ethanol (0.6:0.4) wash solution and 500 μl of 75% ethanol, air dried to remove the ethanol and dissolved in 30 μl 10 mM Tris-HCl (pH 8.0). DNA was quantified using Fluorometer TD-360 (Turner Designs Inc.) and diluted to a final concentration of 100 ng μl^{-1}.

SSR genotyping

Forty three SSR markers were selected from the MAIZEGDB database (http://www.maizegdb.org/cgi-bin/mappedelements.cgi?type=s&chrom=7) based on repeat unit and bin location in order to provide a uniform coverage of chromosome 7 (Appendix D, Table D.2). Care was taken to avoid as many dinucleotide repeats as possible because of difficulty in allele sizing and to include more markers around the *opaque2* locus (bin no. 7.122) to obtain a better coverage of this area. Thirty nine SSR markers that had fluorescent forward primers labeled at 5' end with either 6-carboxyfluorescein (6-FAM) or hexachloro-6-carboxyfluorescein (HEX) purchased synthesized by Genosys, USA were used for sizing alleles with ABI Prism 3100 DNA sequencing system (Applied Biosystems, Foster City, CA). To check the error rate for allele sizing 4 inbreds were duplicated making a total of 96 samples. PCR reactions were performed in a 96 well plate with 10μl volume/well containing 10 ng template DNA, 1X PCR buffer, 0.8 μl of 25mM MgCl2, 1.0 μl 10 mM dNTPs, 0.08 μl of Taq DNA polymerase, 1.0 μl of 2 pM of primer pair and amplified in a GeneAmp PCR system 2700 (Applied Biosystems) with amplification conditions of 94^0C for 2 min; followed by 40 cycles of 94^0C for 30 s, X^0C for 1 min, and 72^0C for 1 min; followed by extension at 72^0C for 20 min. The X stands for annealing temperatures used for amplification for

individual markers, which was determined either from published sources or using Primer Express software. Expected sizes of alleles were determined from published sequence information on MAIZEGDB (http://www.maizegdb.org). A 5μl sample of PCR product was checked on 2% agarose gel for the presence of band. Successful PCR reactions (1.0 μl) from both FAM and HEX were multiplexed with ROX-500 internal size standard on an ABI Prism 3100 DNA sequencer and analyzed by Genotyper version 3.6 (Applied Biosystems). Four SSR markers labeled with IR fluorescent dyes were run on a dual-dye LI-COR 4200 IR^2 gel detection system (LI-COR Inc., Lincoln, NE) and bands scored visually.

Data analyses

Data were transformed to a binary code based on the presence (1) or absence (0) of each allele with columns representing the inbreds and rows the different SSR markers. The resulting matrix was analyzed with NTSYS-pc version 2.1 software package (Exeter Software, Setauket, NY) to estimate the genetic similarities among all pairs of inbreds using Dice's coefficient of similarity as follows:

$$GS_{ij} = 2\ N_{ij}/(N_i + N_j),$$

where N_{ij} is the number of alleles (scored bands) shared by lines i and j, and N_i and N_j are the total number of scored bands in lines i and j, respectively. A dendrogram on the basis of similarity matrix was generated following unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973). Estimates of confidence limits for the grouping were performed with 1000 boostrap resamplings using Winboot (Yap and Nelson, 1996). The polymorphic index content (PIC) for each SSR marker was calculated as

$$PIC = 1 - \Sigma p_i^2$$

where p_i^2 is the frequency of the ith allele in a locus with i alleles.

A graphic display of chromosome 7 linkage group for all 92 inbreds was generated by Supergene[TM] software. Alleles obtained for each SSR marker were numbered 1-n with missing values assigned a zero. Each marker loci was color-coded according to their numerical score.

Pairwise LD values were calculated as the square of the standardized measure (r^2) on excel spreadsheet as:

$$r^2 = D_{ab}{}^2/(p_A+p_B+p_a+p_b)$$

where, D_{ab} is calculated as

$$D_{ab} = (p_{AB} - p_A p_B).$$

Calculated r^2 values were plotted against map distance in cM to display graphically the pattern of LD on the short arm and a comparative distance on long arm of maize chromosome 7.

Parental contributions for derived lines was estimated (Bernardo et al., 2000) as:

$$\lambda_a = (S_{ai} - S_{bi} S_{ab})/ 1- (S_{ab})^2 \text{ and}$$
$$\lambda_b = (S_{bi} - S_{ai} S_{ab})/ 1- (S_{ab})^2$$

where, S_{ai} and S_{bi} are the marker similarities between inbreds a and b with their progeny i, and S_{ab} is the marker similarity between the two inbreds a and b.

RESULTS AND DISCUSSION

Genetic diversity and classification of maize inbreds

The 43 SSR loci identified a total of 200 alleles in the 92 inbreds studied with an average number of 4.7 alleles per locus (a/l) and a range of 2 -17 (Table 4.3). The number of alleles identified for the different SSR loci varied widely. Single sequence repeat loci with dinucleotide, trinucleotide, tetranucleotide and more complex nucleotide

Table 4.3. Single Sequence Repeats (SSRs, types of repeat motif, map positions, number of alleles identified and the polymorphic information content (PIC).

SSR	Repeat Sequence type and no.	Map position	No. of alleles	PIC
	Dinucleotides			
p-bnlg2132	(AG)21	7.0533	9	0.48
p-bnlg1292	(AG)14	7.0795	6	0.70
p-umc1159	(AG)8	7.0920	4	0.59
p-umc2160	(AG)10	7.1185	4	0.09
p-bnlg1367	(AG)42	7.1278	4	0.28
p-bnlg2160	(AG)27	7.1286	5	0.45
p-bnlg1200	(AG)24	7.1424	3	0.47
p-umc1016	(CT)25	7.1558	7	0.60
p-umc1138	(AC)6	7.2477	2	0.05
p-mmc0411	(CT)29	7.2927	9	0.72
p-bnlg1805	(AG)29	7.3921	17	0.85
p-bnlg1666	(AG)34	7.4305	15	0.84
p-bnlg2259	(AG)17	7.4892	6	0.70
p-bnlg2328	(AG)33	7.5174	2	0.16
Average			**7**	**0.50**
	Trinucleotides			
p-umc1480	(GAA)4	7.0200	3	0.56
p-umc2364	(GGA)7	7.1077	3	0.52
p-umc2392	(GGC)5	7.1147	2	0.02
p-phi057	(GCC)4	7.1224	3	0.05
p-umc1401	(CCA)4	7.1530	4	0.49
p-phi034	(CCT)4	7.1799	4	0.60
p-umc2098	(CAG)5	7.2000	2	0.41
p-umc2142	(AGG)4	7.2463	4	0.61
p-umc1787	(CGG)4	7.2524	2	0.16
p-umc1585	(TGG)7	7.2636	4	0.68
p-umc1567	(AGA)4	7.3084	6	0.62
p-umc1408	(CGG)5	7.3654	3	0.54
p-umc1134	(AGC)7	7.3812	3	0.04
p-umc1710	(CTG)5	7.4105	4	0.64
p-umc1782	(GAC)4	7.4539	3	0.20
p-phi328175	AGG	7.4729	5	0.66
p-phi069	GAC	7.5452	3	0.55
p-phi051	AGG	7.64414	4	0.61
Average			**3**	**0.44**
	Tetranucleotides			
p-umc1545	(AAGA)4	7.0055	4	0.52
p-umc2327	(TCTC)5	7.158	6	0.64
p-umc1456	(AACC)5	7.33424	7	0.74
p-phi114	(GCCT)3	7.37161	4	0.47
p-umc1406	(CTCA)4	7.5989	3	0.33
p-phi116	ACTG/ACG***	7.61150	7	0.67
Average			**5**	**0.56**
	Higher Repeats			
p-umc1241	(GTCTTTG)4	7.0138	3	0.38
p-umc1426	(AGAGG)4	7.0478	3	0.35
p-umc1066	(GCCAGA)5	7.12245	3	0.06
p-umc1577	(CTTGGC)4	7.1252	2	0.07
p-umc1068	(GAAAA)6(GAA)2	7.132	3	0.15
Average			**3**	**0.20**

repeat motifs identified an average of 6.64 a/l (range 2-17), 3.33 (range 2-6), 4.8 a/l (range 3-7) and 2.8 a/l (range 2-3), respectively. Markers p-bnlg1805 and p-bnlg1666, that were both dinucleotide repeats showed 17 and 15 alleles, respectively. Previous studies have reported similar averages of 4.9 a/l among 40 U.S. maize inbreds analyzed by 83 SSR loci (Lu and Bernardo, 2001), 5.0 a/l among 94 temperate inbreds utilizing 70 SSR loci (Senior et al., 1998) and 5.9 a/l (Dubreuil et al., 1996). Liu et al. (2003) reported an average of 21.7 a/l in a study of 260 diverse maize inbred lines including temperate, tropical, popcorn and sweet corn lines using 94 SSR loci. The discriminatory power of the markers measured as PIC showed average values of 0.50 (range 0.05 – 0.84), 0.44 (range 0.02 – 0.68), 0.56 (range 0.33 – 0.74), and 0.20 (range 0.06 – 0.38) for di, tri, tetra and more complex repeat motifs, respectively. In general, dinucleotide repeat motifs have been reported to identify a greater proportion of alleles and a higher PIC value due to higher number of repeat motifs and frequent indels in the flanking sequences (Smith et al., 1997; Senior et al., 1998; Liu et al., 2003). However, a comparison of dinucleotide repeats arranged according to their map positions with number of alleles (Fig. 4.2) and PIC values (Fig. 4.3) showed lower number of alleles and lower PIC values around the *opaque-2* region (Bin 7.122). These results support the intense selection pressure for the *opaque-2* gene and characteristics for modified endosperm expression during the development of high lysine maize germplasm that define this unique set of inbreds. In a study of genetic diversity under stress and non stress environments, Betran et al. (2003f) reported reduced diversity in chromosomal segments associated with QTL's identified for plant response to drought stress due to effects of selection and genetic drift.
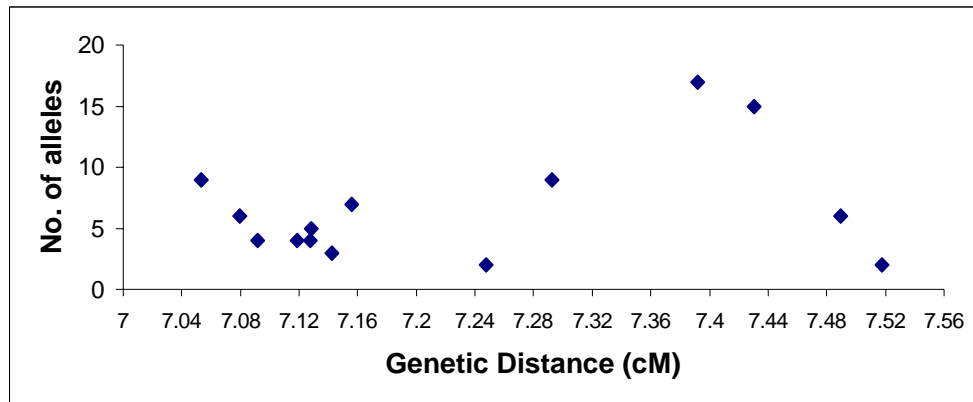
Fig. 4.2. Relationship of genetic distance and number of alleles for dinucleotide SSR markers.
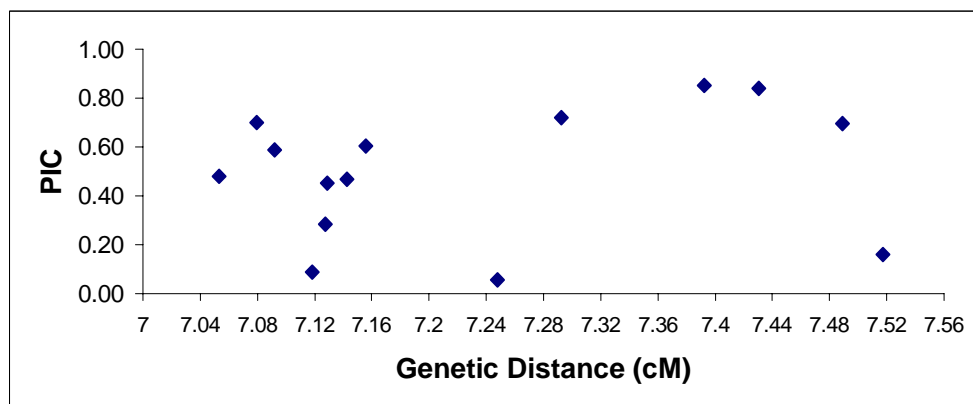
Fig. 4.3. Relationship of genetic distance and polymorphic information content (PIC) for dinucleotide SSR markers.

A dendrogram for all 92 high lysine maize inbreds studied utilizing 43 SSR loci based on their GS_{ij} estimates identified four major groups that were in agreement with the known pedigrees of these lines (Fig. 4.4). Initially, the choice of selection of SSR loci was done on the basis of their bin number to get a uniform coverage of chromosome 7. Greater density of markers were used around the *opaque-2* locus than in the rest of the chromosome to get a better coverage of that region. The first cluster comprised all eight inbreds derived from population 69 that were almost identical in their allelic composition (average GS > 0.95). Two inbreds derived from tropical lines G26Qc x CML 161 and one inbred derived from population 65 grouped together with population 69 (average GS = 0.70), which is in agreement with their origin and past selection history. The second cluster grouped most of the NSS temperate germplasm that included eight NSS early and nine NSS late germplasm. However, inbreds NSE196B3 and NSE196B1 that were NSS in origin grouped with SS late germplasm. Several factors could be responsible for this discrepancy, such as complex breeding history of temperate maize inbreds (Gerdes et al., 1993), often inaccurate or incomplete pedigree information (Liu et al., 2003), and selection and genetic drift during inbreeding. The third cluster comprised of inbreds derived from two-way and multiple crosses between temperate QPM lines (Tx802 and Tx806), temperate opaque lines (B73o2 and Mo17o2), subtropical CIMMYT inbred CML 161, subtropical South African inbreds (Do940y and Ko326y) and temperate non QPM inbreds (B97, B104, Tx714, NC300 and Tx770). Despite their complex pedigrees there was clear differentiation between this cluster of derived lines from other major clusters, which reflects their unique characteristics and allelic composition, having been derived from introgression of subtropical germplasm into temperate germplasm, but adapted to southern U.S. environments. Inbred derived from population 70 and temperate x tropical high oil QPM grouped loosely with derived lines(GS<0.55). Similarly, inbreds Tx802 and a derived line from a cross between (Do940y x Tx802) that were almost identical (GS > 0.95), together grouped loosely with
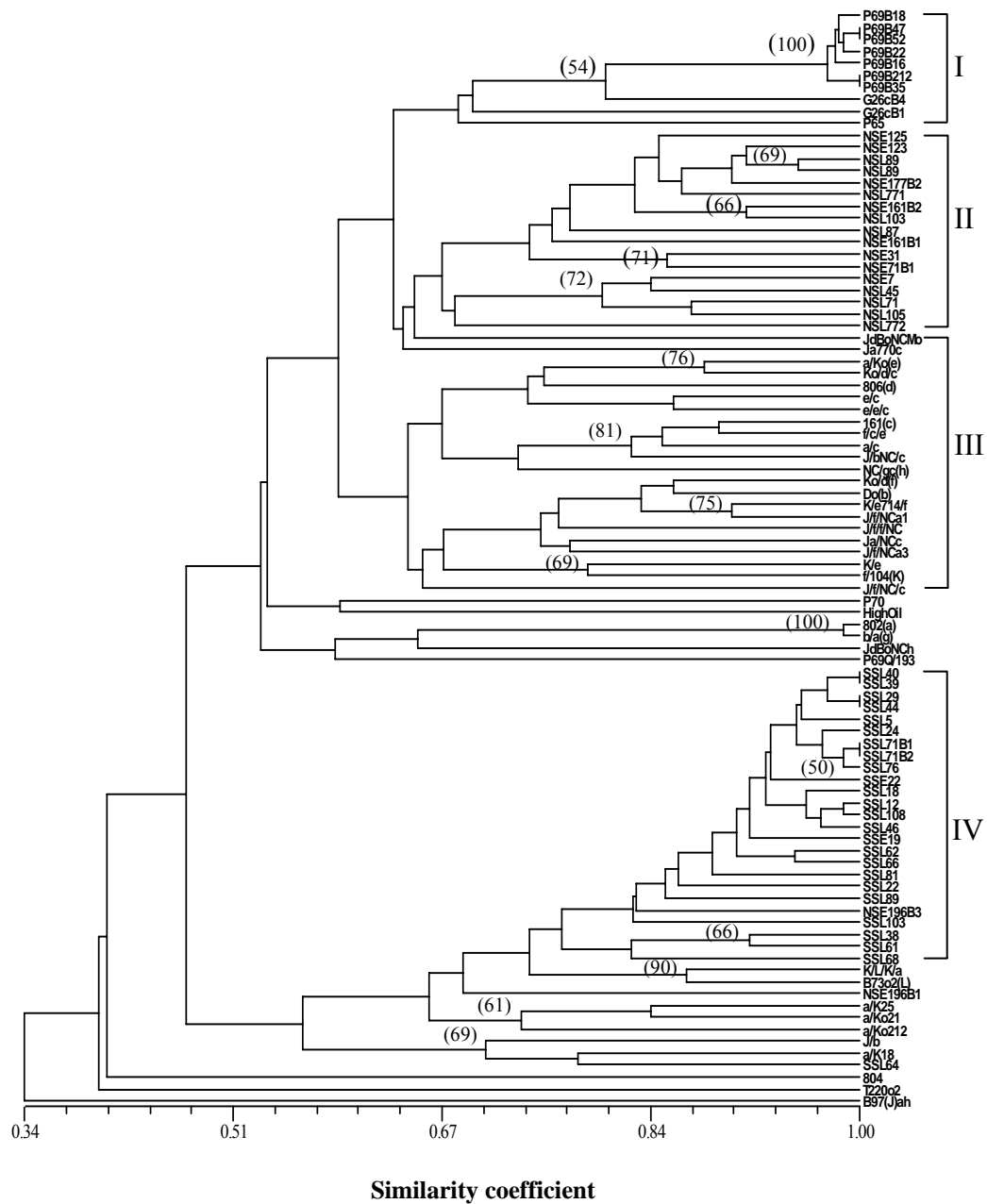
Fig. 4.4. Dendrogram of 92 high lysine maize inbreds as revealed by cluster analysis of genetic similarities of 43 SSR markers.

high oil and population 70 inbreds. One inbred derived from a complex cross involving multiple lines [B97, Tx806, Tx802, inbreds from South Africa (Bo395y and Do940y) and NC300] grouped loosely with the previous two inbreds, Tx802 and inbred derived from (Do940y x Tx802). The fourth cluster included 2 SS early inbreds, 23 SS late inbreds and some derived lines from crosses between B73o2, Tx802, B104, B97 and Ko326y. Some inbreds such as Tx804, T220o2, and a derived line involving complex multiple crossing between several inbreds (B97, Tx802, NC300 and Do940y) did not group with any other inbred. Altogether the 43 SSR loci used to characterize all 92 inbreds were highly efficient in discriminating four major clusters in this study that were consistent with known pedigree information and origin. Confidence intervals derived using 1000 permutations of bootstrap analysis were in general moderately high for individuals within major groups except for SS inbreds, where they were very low (< 50%) due to several possibilities of grouping within this cluster that reflects the low inherent genetic diversity within the SS group.

Characterization of haplotypes on chromosome 7

Chromosomal haplotypes of all 92 inbreds based on SSR loci map position from lowest (7.0055) to highest (7.64414) bin numbers and arranged according to their clustering pattern derived from UPGMA dendrogram is illustrated in Fig. 4.5. Twenty two SSR markers were selected on the short arm of chromosome 7 with a high density of markers around the *opaque-2* gene (17 markers placed within a distance of approximately 21 cM), while the remaining 21 markers were selected on the long arm of chromosome 7 providing a uniform coverage. The haplotypes of all 92 inbreds including 17 markers around the *opaque-2* gene (bin no. 7.07952 – 7.2) were expanded to show more clarity in allelic diversity in this region (Fig. 4.6). Haplotypes for inbreds derived from population 69 were extremely identical showing only three different haplotypes that were due to two different alleles (bin no. 7.10765 on the short arm and bin no. 7.5452 on the long arm) out of 43 SSR loci spanning about 640 cM. The other three inbreds derived from tropical lines G26Qc x CML 16 and population 65 showed more

Fig. 4.5. Chromosome 7 haplotypes for 92 high lysine maize inbreds.
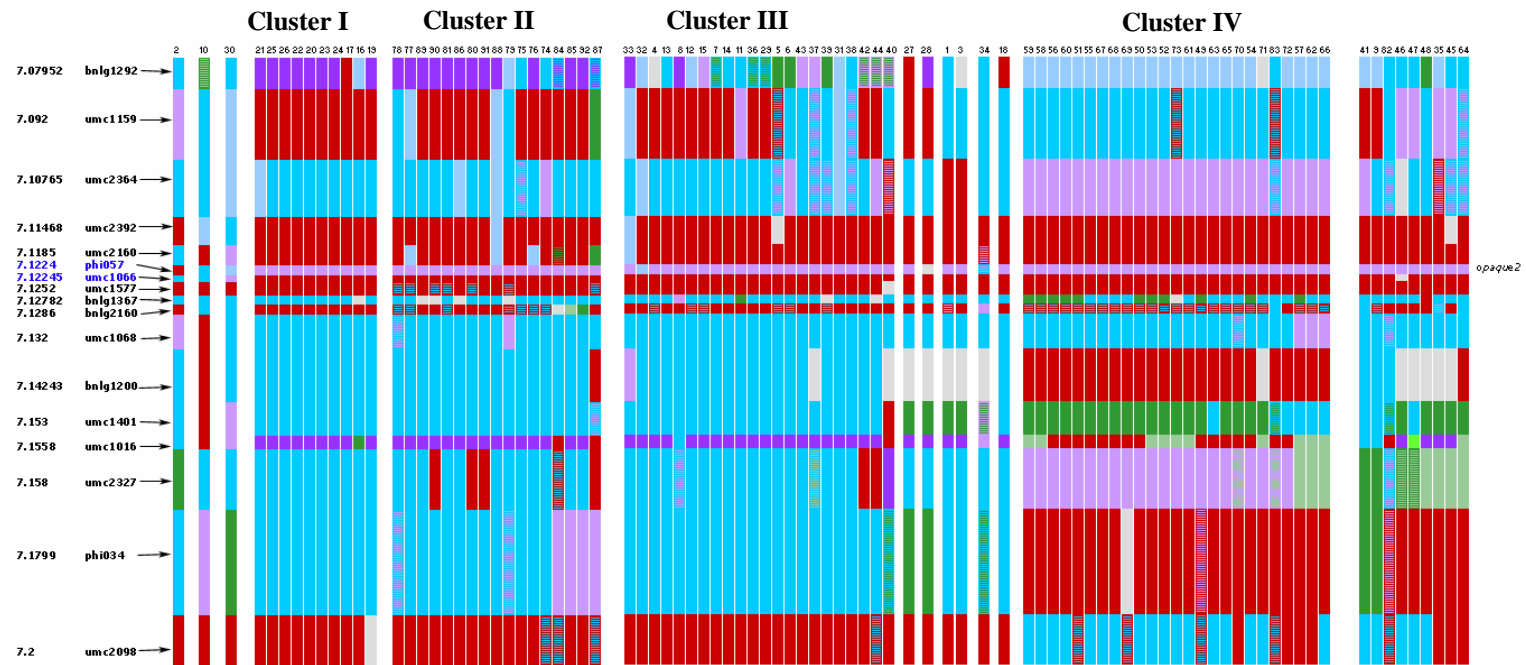
Fig. 4.6. Short arm of chromosome 7 haplotypes for 92 high lysine maize inbreds.

allelic differences that were more prevalent in the top part of the short chromosome (bin no. 7.0055 – 7.07952) and the long arm of chromosome 7. Three different alleles were identified in bin no. 7.07952 and one different allele in bin no. 7.1558 in the region surrounding the *opaque-2* gene (Fig. 4.6).

A comparison of the allelic differences between the NSS and SS clusters was done to determine the genetic diversity between these two groups. In the opaque-2 region (bin no. 7.092 – 7.2) 13/17 NSS haplotypes were different with only 3 predominant types as compared to 6/20 different SS haplotypes with 6 predominant haplotypes (Fig. 4.6). In a corresponding 10 cM window on the long arm of chromosome 7 (bin no. 7.36 – 7.47) there were 11/17 different NSS haplotypes with only 3 predominant haplotypes as compared to 7/20 different haplotypes in the SS cluster with 9 predominant haplotypes (Fig. 4.5). A comparison of the NSS and SS group was also done with respect to the number of alleles per marker at all the loci (Fig. 4.7) and the frequency of the predominant allele per marker (Fig. 4.8). In general in the NSS group more number of alleles occurred for most marker loci (range from 1-5), whereas in the SS group there were fewer alleles identified (range from 1-3) (Fig. 4.7). A great diversity in the pattern of the frequency of predominant alleles was observed per loci between the NSS group and SS group (Fig. 4.8). Some regions of the chromosome 7 showed similar trends of lower frequency of alleles for both SS and NSS groups (markers 3, 14-15, 19-20 and 33-35), whereas contrasting trends were observed in other regions (markers 6, 20-21, 27-28, 36-37). These results indicate that NSS inbreds are more variable than SS inbreds with regards to their allelic compositions in both *opaque-2* region as well as the long arm of chromosome 7. This difference can be the consequence of different selection history and variable degrees of linkage drag associated with the conversions to *opaque-2* versions of both SS and NSS lines. Stiff stalk lines have been selected to perform well with NSS lines and these two groups have reduced allelic diversity in different chromosomal segments. Furthermore, the original narrow genetic base for SS inbreds, mainly originated from B73,B37 and B84 could explain the lower genetic variability of SS inbred in general as compared to NSS inbreds.
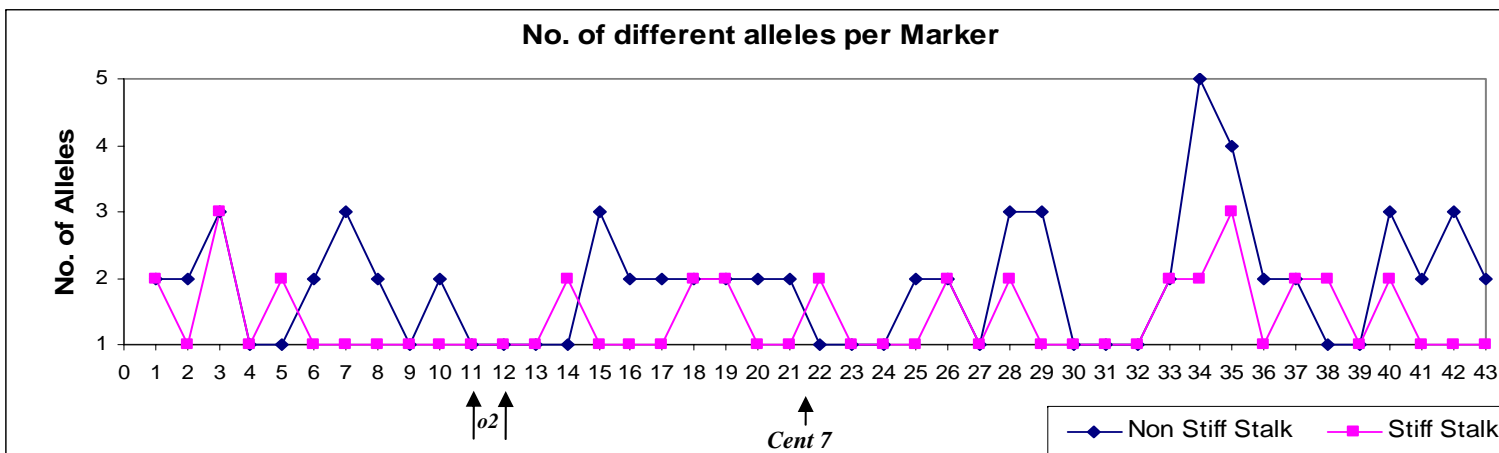
Fig. 4.7. Number of alleles per loci for 43 SSR markers on chromosome 7 of maize.
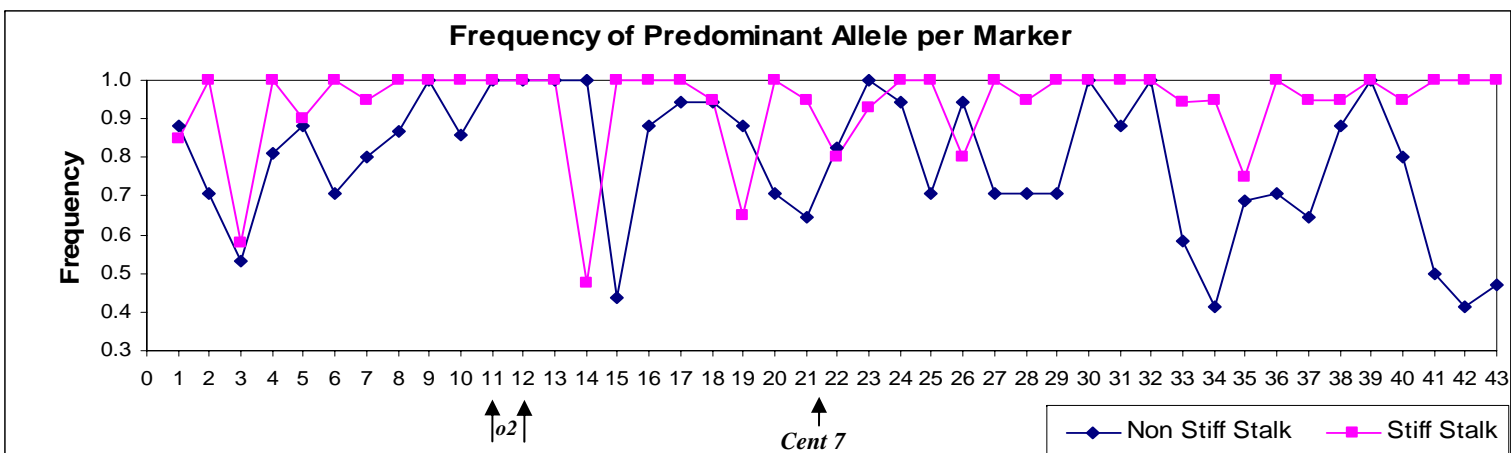
Fig. 4.8. Frequency of predominant alleles per loci for 43 SSR markers on chromosome 7 of maize.

However, this variability is drastically reduced in both NSS and SS inbreds as we move closer to the *opaque-2* gene (bin no. 7.122) with only 5 different alleles identified across all inbreds (Fig. 4.6). Strong selection pressure to maintain the *opaque-2* phenotype has reduced diversity and increases LD in the surrounding region. Persistence of strong LD across at least 5 cM (an order of several hundred megabases) genetic distance indicates a strong selective sweep for the opaque-2 region. Almost similar and striking trend of artificial selection on diversity and LD has been reported by maize *Y1* locus (*y1* allele governs white endosperm color and *Y1* allele yellow endosperm). The yellow allele has been reported to be almost 20 times less diverse than the corresponding white allele, a fact that is consistent with strong continuing selection for the yellow endosperm color (Palaisa et al., 2003; Rafalski and Morgante, 2004) in temperate maize. Romero-Severson et al., (2001) have reported similar observations in a study of genetic similarities and shared haplotypes within closely related groups of elite U.S. and European temperate maize.

The derived lines showed a contrasting trend with 11 different haplotypes in the region around the *opaque-2* gene as compared to 20 different haplotypes in the lomg arm of chromosome 7 with 8 and 3 predominant haplotypes, respectively. These results indicate that the derived lines showed fewer different haplotypes in this chromosome possibly due to selection for alleles of one predominant inbred. Lines derived from crosses between normal wild type *O2* lines and opaque *o2* lines have haplotypes similar to the *o2* lines around the *opaque2* gene. This is consistent with a selection for the opaque phenotype during line development.

Parental contribution in shared haplotypes

A sample of derived lines to estimate parental contribution in shared haplotypes is illustrated in Fig. 4.9. The proportion of common alleles and the parental contributions of inbreds to derived lines are presented in Fig.4.9. The parental contributions of inbreds Tx802, CML 161 to the derived line from the cross of these two parents were 0.06 and 0.82, respectively. In this set CML161 showed similar alleles for 33 markers
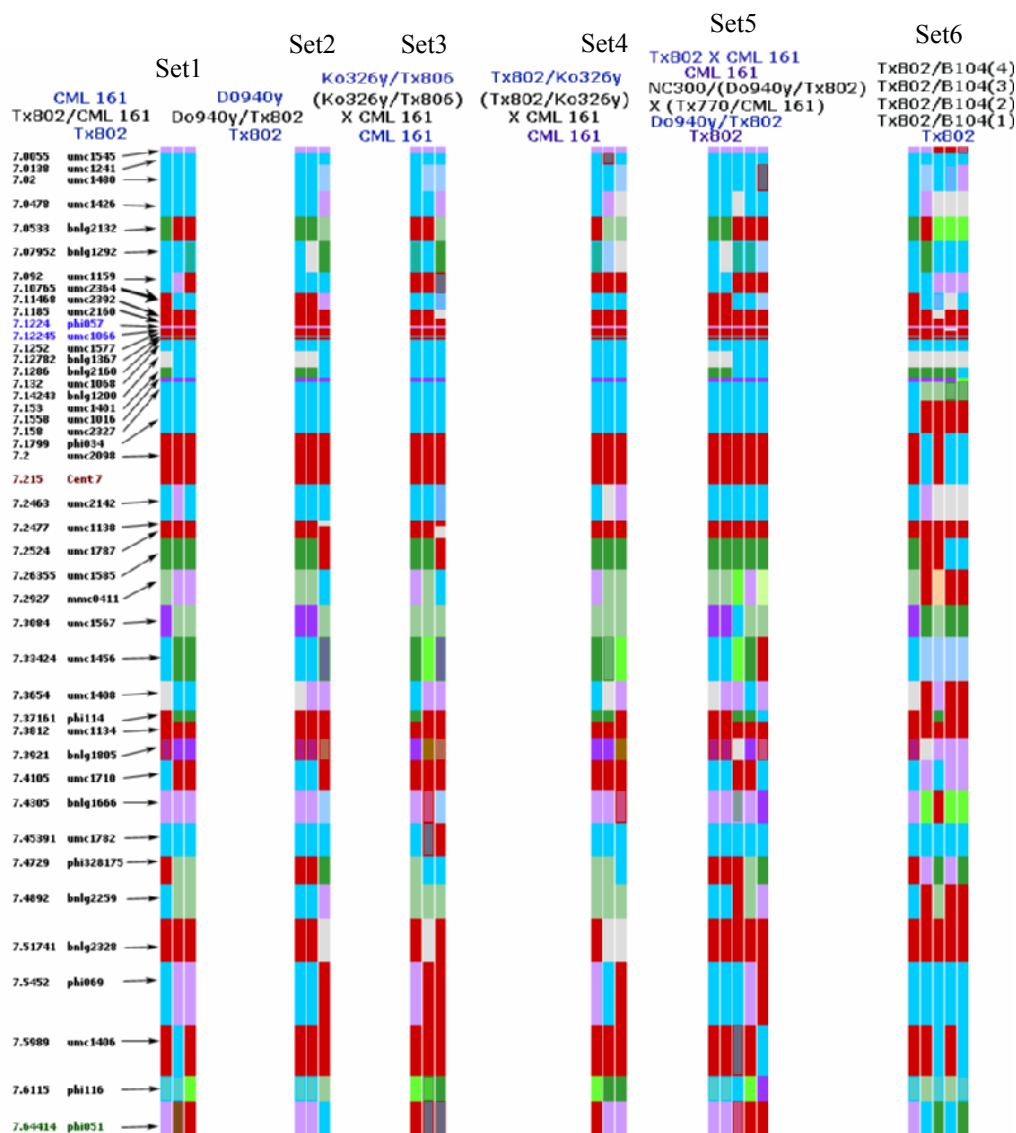
Parental contributions:

Set 1 λ (Tx802 →Tx802/CML161 = 0.06), λ (CML161 →Tx802/CML161 = 0.82)

Set 2 λ (Tx802 →Do940y/Tx802 = 0.92), λ (D0940y →Do940y/Tx802 = 0.06)

Set 3 λ (CML161 →[Ko326y/Tx806]/CML161 = 0.53)

λ (Ko326y/Tx806 →[Ko326y/Tx806]/CML161 = 0.40

Set 4 λ (CML161 →[Tx802/Ko326y]/CML161= 0.46

λ (Tx802/Ko326y →[Tx802/Ko326y]/CML161= 0.46)

Set 5 λ (Do940y/Tx802 →[NC300/[Do940y/Tx802]/[Tx770/CML161]= 0.33)

λ (CML161→[NC300/[Do940y/Tx802]/[Tx770/CML161]= 0.57)

Fig. 4.9. Parental contributions of six sets of inbreds to their derived lines.

out of 43 total markers, predominantly in the long arm of chromosome 7. In the second set the parental contributions of Tx802 and Do940y were 0.92 and 0.06, respectively. Do940y showed similar alleles for 41 markers out of 43 markers. In the third set the parental contribution of CML 161 and (Ko326y/Tx806) was 0.53 and 0.40, respectively. In the fourth set the parental contributions of CML161 and (Tx802/Ko326y) were equal being 0.46 for both inbreds. In the fifth set the parental contributions of (Do940y/Tx802) and CML161 were 0.33 and 0.57, respectively. The last set (6) consisted of Tx802 and sister lines derived from cross of Tx802 x B104. Four alleles were identified that were contributed by Tx802 exclusively to at least one of the derived line. The sister lines derived from the same cross showed extensive dissimilarity among themselves, with one sister line (Tx802 x B104[1]) showing 4 alleles that were not present in any other line. Considering intense selection practices resorted to in the development of elite temperate germplasm, fixing segments of chromosomes and consequently alleles, may result in extensive haplotype sharing among inbreds derived from common progenitors (Romero-Severson et al., 2001).

Four alleles were identified in the first set that were contributed by neither parent to the derived line. It is possible that they could have arisen due to *de novo* mutation or were present on a different chromosome but were identified by the same SSR due to vast duplications in segments of chromosomes 2 and 7. Although these results are based on a single chromosome, nevertheless have implications in tracking alleles in derived lines or crosses for quality traits in high lysine germplasm and possibly of greater significance in identifying potential parents for breeding crosses. Tracking parental contributions in parents and their derived inbreds or crosses is a unique method of identifying segments of chromosomes that have undergone fixation due to selection or have been subject to genetic drift, phenomena common in small size breeding populations. Utilizing molecular markers obviates the use of calculating inbred progeny statistics and visibly identifies regions of chromosomes that are actually transferred from one parent to the progeny. SSR markers are especially suitable for this study owing to their properties of high reproducibility and abundant polymorphism in plant species, especially maize.

Linkage disequilibrium

Linkage disequilibrium calculated as square of the standardized measure (r^2) were plotted against map distance in cM to display graphically the pattern of LD on the short arm and a comparative distance on long arm of maize chromosome 7 (Fig. 4.10). In the short arm of chromosome 7, LD was found to increase exponentially (range 0.01 – 0.9) as genetic distance increased from 7.05 cM to 7.12 cM. Specifically, in the opaque-2 region (7.10 cM to 7.122 cM) r^2 values were beyond the expected range of 0 to 1, as the markers were almost monomorphic. Linkage disequilibrium values decreased rapidly in the cM range 7.15 to 7.18 and then increased slightly towards the centromeric region of the chromosome.

In contrast in the long arm of chromosome 7 LD values in general remained low (< 0.31) for a considerable range of genetic distance. Linkage disequilibrium values increased rapidly to a maximum of 0.48 near the telomeres of the long arm of chromosome 7, where the gamma-zein gene *gzr1* is mapped. These results indicate different degrees of LD along chromosome 7, which is consistent with intense selection pressure for *o2* (in the opaque-2 region) and possibly for genes involved in endosperm modification.

Linkage disequilibrium pattern is largely governed by recombination and decays with genetic distance. However, many historical, selection bottleneck, genetic drift, introgression of germplasm, and population genetic factors also influence LD (Nordborg and Tavare, 2002). Studies to evaluate and understand the patterns of LD in plant genomes have attracted recent interest because it controls the resolution and practicability of association-mapping studies (Remington et al., 2001). Literature suggests that increased LD in selected regions of genome can be efficiently used to pinpoint selective sweeps even in the absence of selective signatures detectable by traditional selection tests (Saunders et al., 2002). Although in our study there was little or no evidence that LD decayed rapidly within a small range of genetic distance, the pattern of LD observed was consistent with the intense selection of opaque-2 and modified opaque-2 genes. It may be further possible to conduct association genetics

using phenotypic data for endosperm modification ratings and LD estimates to tentatively map important genes on maize chromosome 7 affecting this trait (Flint-Garcia et al., 2003).
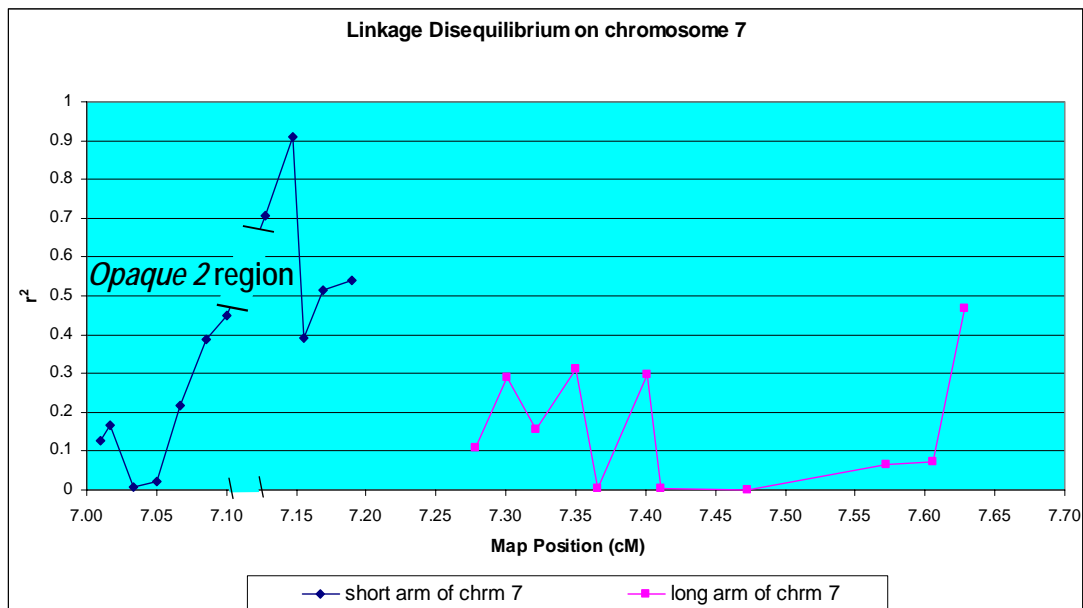
Fig. 4.10. Pattern of linkage disequilibrium comparing short arm and long arm of maize chromosome 7.

CHAPTER V

SUMMARY AND CONCLUSIONS

EXPERIMENT 1: COMBINING ABILITIES OF QUALITY PROTEIN MAIZE INBREDS

QPM hybrids yielded less than commercial checks. Although GCA effects across environments were non-significant for grain yield but were highly significant for agronomic and kernel quality traits. Based on GCA effects, TAMU inbreds had earlier maturities, shorter plants, and less grain moisture content than more subtropical CIMMYT and SA inbreds. The best yielding hybrids and highest SCA effects resulted from crosses among inbreds from different programs: TxX124 x CML 176, Tx811 x CML 181, Bo59w x CML 184 among the white hybrids, and Tx802 x Do940y among the yellow hybrids. It was concluded that QPM inbreds developed in different programs could represent potential heterotic groups for use in hybrid development and introgression of germplasm.

EXPERIMENT 2: AGRONOMIC, AFLATOXIN AND QUALITY ANALYSIS OF HIGH LYSINE MAIZE INBREDS AND TESTCROSSES

Repeatibilities for grain yield were in general high per environment and across all environments. Population 69 inbreds developed at CIMMYT that have a flinty orange texture were least susceptible to afaltoxin accumulation both in inbreds and testcrosses at all locations. Aflatoxin in testcrosses was positively correlated with endosperm texture (0.67) and kernel integrity (0.60) but negatively correlated with grain yield (-0.30) and silking date (-0.50). Tryptophan and methionine content of QPM inbreds and hybrids revealed a negative correlation with endosperm translucence, which is a measure of kernel hardness. In general the *o2/o2* germplasm had lower methionine levels than the wild type germplasm regardless of kernel hardness, suggesting that methionine levels

could be reduced by the *o2/o2* mutation. Evaluations of inbreds testcrossed to Tx804 revealed significant correlations of the amino acid levels of inbred lines with those of the hybrids, although the predictive value was low (R^2= 0.13 and 0.27 for methionine and tryptophan, respectively). The reduction in tryptophan during conversion to the hard-kernel phenotype and the reduction in methionine in *o2* germplasm both reduce the nutritional value of QPM. It may be possible to correct these deficiencies by selection for tryptophan and methionine levels during breeding.

EXPERIMENT 3: GENETIC DIVERSITY AND HAPLOTYPING OF MAIZE CHROMOSOME 7 IN *OPAQUE-2* HIGH LYSINE INBREDS

Evaluation of genetic diversity among 92 inbreds using SSR markers detected overall 200 alleles with an average of 4.7 alleles/locus (range 2 to 17). Dendrograms of genetic similarity showed clusters in agreement with the different origin of inbreds. In general, inbreds having common origin shared great proportion of similar haplotypes. Haplotypes around the *opaque-2* locus were more similar between donor and converted lines. Significant linkage disequilibrium was detected around *opaque-2* locus spanning several cMs suggesting high selection pressure during the conversion of normal lines to *opaque-2*. Estimation of parental contribution identified haplotypes segments of chromosome 7 that were exclusively contributed by one or the other parent and showed high estimates. These results can be useful in parental selection to create breeding populations that enhance genetic variation along chromosome 7, and identification of parental inbreds that maximize heterozygosity in hybrid combinations.

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APPENDIX A

SAS program for running the analysis for biplot for grain yield for white and yellow diallel crosses using mean grain yield across locations to visualize relationships among parental inbreds in hybrid combinations.

```
OPTIONS PS=500 LS=78;
DATA DLL;
INFILE 'C:/sandy/sas data/GYdata.prn/' FIRSTOBS=2;
INPUT ENTRY $ A B C D E F G H I;
PROC PRINT;
PROC PRINCOMP COV OUT=PCAOUT N=2;
PROC PRINT;
ID ENTRY;
VAR PRIN1 PRIN2;
RUN;
```

The SAS System Output

Obs	ENTRY	A	B	C	D	E	F	G	H	I
1	A	6.107	6.048	6.566	5.998	5.568	6.074	6.608	6.214	5.902
2	B	6.048	5.864	6.454	6.138	5.780	6.428	4.716	6.190	5.572
3	C	6.566	6.454	6.579	6.232	5.418	6.368	7.508	6.864	6.792
4	D	5.998	6.138	6.232	6.436	6.500	6.594	6.324	7.166	6.424
5	E	5.568	5.780	5.418	6.500	5.907	5.428	6.694	5.914	6.320
6	F	6.074	6.428	6.368	6.594	5.428	6.493	7.236	7.134	6.474
7	G	6.608	4.716	7.508	6.324	6.694	7.236	6.293	5.324	5.976
8	H	6.214	6.190	6.864	7.166	5.914	7.134	5.324	6.379	6.142
9	I	5.902	5.572	6.792	6.424	6.320	6.474	5.976	6.142	6.193

The PRINCOMP Procedure

Observations	9
Variables	9

Simple Statistics

	A	B	C	D	E
Mean	6.120555556	5.910000000	6.531222222	6.423555556	5.947666667
StD	0.320248775	0.532984990	0.558763764	0.334574024	0.464537942

Simple Statistics

	F	G	H	I
Mean	6.469888889	6.297666667	6.369666667	6.199444444
StD	0.535994507	0.876972348	0.599186949	0.358275422

```
                            Covariance Matrix


                  A               B               C               D               E

A    0.1025592778    -.0273012500    0.1420034861    -.0112234722    0.0049527083
B    -.0273012500    0.2840730000    -.1418685000    0.0322495000    -.1871482500
C    0.1420034861    -.1418685000    0.3122169444    0.0039246111    0.0942692083
D    -.0112234722    0.0322495000    0.0039246111    0.1119397778    0.0158708333
E    0.0049527083    -.1871482500    0.0942692083    0.0158708333    0.2157955000
F    0.1196288194    -.0828242500    0.2592421528    0.0678231944    0.1076442083
G    0.0451029583    0.1320262500    -.1029925417    -.0555336667    -.1341708750
H    -.0131410417    0.2775645000    -.1126685417    0.0298925833    -.1221163750
I    0.0117143472    0.0925997500    -.0523554861    0.0288302222    -.0358132083


                            Covariance Matrix


                  F               G               H               I

A    0.1196288194    0.0451029583    -.0131410417    0.0117143472
B    -.0828242500    0.1320262500    0.2775645000    0.0925997500
C    0.2592421528    -.1029925417    -.1126685417    -.0523554861
D    0.0678231944    -.0555336667    0.0298925833    0.0288302222
E    0.1076442083    -.1341708750    -.1221163750    -.0358132083
F    0.2872901111    -.1524280417    -.0232245417    -.0271358194
G    -.1524280417    0.7690805000    0.1825963750    0.2484570417
H    -.0232245417    0.1825963750    0.3590250000    0.1275340417
I    -.0271358194    0.2484570417    0.1275340417    0.1283612778


                  Total Variance    2.5703413889

                Eigenvalues of the Covariance Matrix


           Eigenvalue    Difference    Proportion    Cumulative

      1    1.21508539    0.64000614       0.4727        0.4727
      2    0.57507925                     0.2237        0.6965


                            Eigenvectors


                       Prin1        Prin2

                  A    -.054928     0.326007
                  B    0.355852     -.245106
                  C    -.308829     0.497258
                  D    -.027015     -.030634
                  E    -.268628     0.158885
                  F    -.281225     0.370787
                  G    0.646039     0.617385
                  H    0.377176     -.101968
                  I    0.251734     0.176492


                       The SAS System
```

ENTRY	Prin1	Prin2
A	0.33083	-0.06006
B	-1.17153	-1.15312
C	1.44801	0.72099
D	0.37056	-0.13654
E	0.74428	-0.78452
F	1.32939	0.24731
G	-1.62023	1.40728
H	-0.84601	-0.26676
I	-0.58530	0.02542

APPENDIX B

Table B.1. QPM testcrosses between high lysine maize inbreds with different origins and Tx804 and hybrid checks evaluated in 2002 for agronomic evaluation and aflatoxin resistance.

Entry	Pedigree
1	Pop. 65 Yellow Flint QPM-B-B-B1-3/TX804
2	Pop. 65 Yellow Flint QPM-B-B-B4-1/TX804
3	Pop. 66 Yellow Dent QPM-B-B-B3-1/TX804
4	Pop. 66 Yellow Dent QPM-B-B-B4-2/TX804
5	Pop. 69 Templado Amarillo QPM-B-B-B1-1/TX804
6	Pop. 69 Templado Amarillo QPM-B-B-B2-2/TX804
7	Pop. 69 Templado Amarillo QPM-B-B-B2-7/TX804
8	Pop. 69 Templado Amarillo QPM-B-B-B2-10/TX804
9	Pop. 69 Templado Amarillo QPM-B-B-B3-5/TX804
10	Pop. 69 Templado Amarillo QPM-B-B-B3-6/TX804
11	Pop. 69 Templado Amarillo QPM-B-B-B3-10/TX804
12	Pop. 69 Templado Amarillo QPM-B-B-B4-2/TX804
13	Pop. 69 Templado Amarillo QPM-B-B-B4-7/TX804
14	Pop. 69 Templado Amarillo QPM-B-B-B4-11/TX804
15	Pop. 69 Templado Amarillo QPM-B-B-B5-4/TX804
16	Pop. 69 Templado Amarillo QPM-B-B-B5-7/TX804
17	Pop. 69 Templado Amarillo QPM-B-B-B5-12/TX804
18	Pop. 69 Templado Amarillo QPM-B-B-B5-13/TX804
19	Pop. 69 Templado Amarillo QPM-B-B-B6-3/TX804
20	Pop. 69 Templado Amarillo QPM-B-B-B6-8/TX804
21	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-1/TX804
22	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-7/TX804
23	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-10/TX804
24	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-2/TX804
25	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-3/TX804
26	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-4/TX804
27	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-7/TX804
28	Pool 26 Tropical Late Yellow Dent QPM-B-B-B3-1/TX804
29	Pool 26 Tropical Late Yellow Dent QPM-B-B-B8-2/TX804
30	Pool 33 Subtropical Intermediate Yellow Flint QPM-B-B-B1-2/TX804
31	Pool 33 Subtropical Intermediate Yellow Flint QPM-B-B-B2-1/TX804
32	Pool 34 Subtropical Intermediate Yellow Dent QPM-B-1-B-1/TX804
33	Pool 34 Subtropical Intermediate Yellow Dent QPM-B-2-B-1/TX804
34	Pool 34 Subtropical Intermediate Yellow Dent QPM-B-7-B-1/TX804
35	Temperate x Tropical High-Oil QPM-B-3-B-1/TX804
36	Temperate x Tropical High-Oil QPM-B-5-B-1/TX804
37	Temperate x Tropical High-Oil QPM-B-5-B-4/TX804
38	Temperate x Tropical High-Oil QPM-B-6-B-3/TX804
39	Temperate x Tropical High-Oil QPM-B-6-B-8 Floury/TX804
40	Temperate x Tropical High-Oil QPM-B-6-B-9 Floury/TX804
41	Temperate x Tropical High-Oil QPM-B-7-B-4/TX804
42	Temperate x Tropical High-Oil QPM-B-7-B-5/TX804
43	Temperate x Tropical High-Oil QPM-B-7-B-9/TX804
44	Temperate x Tropical High-Oil QPM-B-8-B-1/TX804
45	Temperate x Tropical High-Oil QPM-B-8-B-5/TX804
46	Temperate x Tropical High-Oil QPM-B-8-B-6/TX804
47	Temperate x Tropical High-Oil QPM-B-8-B-7/TX804
48	Temperate x Tropical High-Oil QPM-B-8-B-9/TX804
49	Do940y-B/TX804

Table B.1. Continued.

Entry	Pedigree
50	CML161-B/TX804
51	Do940y/Tx802/TX804
52	Do940y x Tx802
53	SR470
54	SL53
55	SR660
56	P31B13
57	P32R25
58	RX897
59	DK668
60	DK687

For Aflatoxin evaluation an additional hybrid (CML161 x CML170) was tested instead of DK 667.

Table B.2. Characteristics of environments used to evaluate QPM testcrosses between inbreds with different origins and Tx804 and hybrid checks in 2004.

Locations	Code	Latitude	Elevation (m)	Plot area (m²/plot)	Water regime
COLLEGE STATION, TX	CS	30°37'	96	9.50	Irrigated
WESLACO, TX	WE	26°09'	22.5	11.85	Irrigated
CASTROVILLE, TX	CA	29°21'	228.2	14.76	Irrigated
BARDWELL, TX	BA	32°17'	126.4	14.78	Rainfed
WHARTON, TX	WH	29°17'	30.3	13.86	Rainfed
GRANGER, TX	GR	30°42'	172.4	15.00	Rainfed
SPRINGLAKE, TX	SP	34°13'	1122.3	12.60	Rainfed

APPENDIX C

Table C.1. QPM lines (inbreds 1) evaluated for endosperm modification, average methionine and average tryptophan of inbred lines evaluated in 2002.

Entry	Inbred lines Pedigree from Selfing	End. Mod. Rating	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
1	Pop. 65 Yellow Flint QPM-B-B-B1-3-B-B	5.0	0.11	0.29
2	Pop. 65 Yellow Flint QPM-B-B-B2-4-B	5.0	0.10	0.40
3	Pop. 65 Yellow Flint QPM-B-B-B4-1-B-B	5.0	0.10	0.41
4	Pop. 65 Yellow Flint QPM-B-B-B4-2-B-B	5.0	0.13	0.33
5	Pop. 66 Yellow Dent QPM-B-B-B3-1-B-B	5.0	0.12	0.39
6	Pop. 66 Yellow Dent QPM-B-B-B4-2-B-B	5.0	0.13	0.40
7	Pop. 69 Templado Amarillo QPM-B-B-B1-1-B-B	5.0	0.12	0.42
8	Pop. 69 Templado Amarillo QPM-B-B-B1-6-B-B	5.0	0.12	0.47
9	Pop. 69 Templado Amarillo QPM-B-B-B1-8-B-B	5.0	0.13	0.46
10	Pop. 69 Templado Amarillo QPM-B-B-B2-2-B-B	5.0	0.13	0.40
11	Pop. 69 Templado Amarillo QPM-B-B-B2-7-B-B	5.0	0.13	0.49
12	Pop. 69 Templado Amarillo QPM-B-B-B2-10-B-B	5.0	0.13	0.44
13	Pop. 69 Templado Amarillo QPM-B-B-B3-1-B-B	5.0	0.12	0.39
14	Pop. 69 Templado Amarillo QPM-B-B-B3-5-B-B	5.0	0.12	0.36
15	Pop. 69 Templado Amarillo QPM-B-B-B3-6-B-B	5.0	0.12	0.43
16	Pop. 69 Templado Amarillo QPM-B-B-B3-7-B-B	5.0	0.13	0.42
17	Pop. 69 Templado Amarillo QPM-B-B-B3-8-B-B	5.0	0.13	0.41
18	Pop. 69 Templado Amarillo QPM-B-B-B3-9-B-B	5.0	0.12	0.39
19	Pop. 69 Templado Amarillo QPM-B-B-B3-10-B-B	5.0	0.11	0.43
20	Pop. 69 Templado Amarillo QPM-B-B-B4-2-B-B	5.0	0.13	0.39
21	Pop. 69 Templado Amarillo QPM-B-B-B4-4-B-B	5.0	0.12	0.39
22	Pop. 69 Templado Amarillo QPM-B-B-B4-7-B-B	5.0	0.13	0.44
23	Pop. 69 Templado Amarillo QPM-B-B-B4-9-B-B	5.0	0.12	0.42
24	Pop. 69 Templado Amarillo QPM-B-B-B4-11-B-B	5.0	0.12	0.40
25	Pop. 69 Templado Amarillo QPM-B-B-B5-4-B-B	5.0	0.12	0.38
26	Pop. 69 Templado Amarillo QPM-B-B-B5-5-B-B	5.0	0.13	0.46
27	Pop. 69 Templado Amarillo QPM-B-B-B5-6-B-B	5.0	0.13	0.43
28	Pop. 69 Templado Amarillo QPM-B-B-B5-7-B-B	5.0	0.12	0.38
29	Pop. 69 Templado Amarillo QPM-B-B-B5-12-B-B	5.0	0.12	0.38
30	Pop. 69 Templado Amarillo QPM-B-B-B5-13-B-B	5.0	0.13	0.45
31	Pop. 69 Templado Amarillo QPM-B-B-B6-3-B-B	5.0	0.13	0.40
32	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-6	5.0	0.11	0.42
33	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-7	5.0	0.12	0.37
34	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-8	5.0	0.11	0.43
35	Pop. 70 Templado Amarillo Dentado QPM-B-B-B2-10	5.0	0.11	0.42
36	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-2	5.0	0.11	0.42
37	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-3	5.0	0.12	0.42
38	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-4	5.0	0.12	0.43
39	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-5	5.0	0.12	0.44
40	Pop. 70 Templado Amarillo Dentado QPM-B-B-B3-7	5.0	0.10	0.38
41	Pool 26 Tropical Late Yellow Dent QPM-B-B-B2-1-B	5.0	0.10	0.36
42	B73 o2-B	1.0	0.13	0.64
43	CML161-B-B	1.0	0.10	0.30
44	Pool 26 Tropical Late Yellow Dent QPM-B-B-B8-2-B-B	3.0	0.10	0.42
45	Pool 33 Subtropical Intermediate Yellow Flint QPM-B1-2	5.0	0.11	0.48
46	Pool 33 Subtropical Intermediate Yellow Flint QPM-1-4	5.0	0.11	0.39
47	Pool 33 Subtropical Intermediate Yellow Flint QPM-B2-1	5.0	0.12	0.41
48	Pool 34 Subtropical Intermediate Yellow Dent QPM-1-1	5.0	0.12	0.42

Table C.1. Continued.

Entry	Inbred lines Pedigree from Selfing	End. Mod. Rating	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
49	Pool 34 Subtropical Intermediate Yellow Dent QPM-1-2	4.7	0.11	0.48
50	Pool 34 Subtropical Intermediate Yellow Dent QPM-1-3	3.6	0.11	0.39
51	Pool 34 Subtropical Intermediate Yellow Dent QPM-2-1	5.0	0.10	0.36
52	B73 o2	1.0	0.13	0.63
53	Temperate x Tropical High-Oil QPM-B-3-B-1-B	5.0	0.10	0.49
54	Temperate x Tropical High-Oil QPM-B-5-B-1-B-B	4.6	0.12	0.45
55	Temperate x Tropical High-Oil QPM-B-5-B-3-B-B	3.0	0.12	0.55
56	Temperate x Tropical High-Oil QPM-B-5-B-4-B-B	4.6	0.13	0.56
57	Temperate x Tropical High-Oil QPM-B-6-B-3-B-B	5.0	0.11	0.42
58	Temperate x Tropical High-Oil QPM-B-6-B-8 Floury	3.6	0.13	0.60
59	Temperate x Tropical High-Oil QPM-B-6-B-9 Floury	1.4	0.11	0.54
60	Temperate x Tropical High-Oil QPM-B-7-B-4	2.4	0.10	0.43
61	Temperate x Tropical High-Oil QPM-B-7-B-5	2.3	0.11	0.49
62	Temperate x Tropical High-Oil QPM-B-7-B-9	3.3	0.11	0.48
63	Temperate x Tropical High-Oil QPM-B-8-B-1	4.0	0.09	0.36
64	Temperate x Tropical High-Oil QPM-B-8-B-2	5.0	0.10	0.37
65	Temperate x Tropical High-Oil QPM-B-8-B-4	5.0	0.10	0.39
66	Temperate x Tropical High-Oil QPM-B-8-B-5	4.9	0.09	0.33
67	Temperate x Tropical High-Oil QPM-B-8-B-6-B	3.0	0.10	0.38
68	Temperate x Tropical High-Oil QPM-B-8-B-7	1.0	0.10	0.44
69	Do940y-B-B	2.8	0.11	0.39
70	Temperate x Tropical High-Oil QPM-B-8-B-9-B	5.0	0.49	0.32
71	Tx802-B-B	1.0	0.11	0.43
72	(Tx806 x Bo395y)-5-1-1-1-1-B-B-B-B-B	4.9	0.13	0.49
73	(Tx802 x Ko326y)-18-1-1-1-B-B-B-B-B	5.0	0.12	0.42
74	(Ko326y x Tx806)-2-2-1-1-B-B-B-B-B	5.0	0.11	0.38
75	(Ko326y x Tx806)-6-1-1-1-B-B-B-B-B	1.4	0.12	0.44
76	Do940y-B-B	2.8	0.11	0.37
77	CML161-B-B	1.0	0.11	0.33
78	Tx804-B	2.6	0.12	0.46
79	Tx806-B-B-B	1.0	0.11	0.37
80	B73 o2-B	1.0	0.13	0.64
81	Tx772	5.0	0.13	0.28
82	NC300-B-B	5.0	0.12	0.28
83	Tx601y	5.0	0.13	0.28
84	FRB73	3.0	0.17	0.32
85	B104	5.0	0.21	0.32
86	FR2128	5.0	0.14	0.26
	Mean	**4.2**	**0.12**	**0.42**
	Std Dev	**1.35**	**0.04**	**0.07**
	Correl. End. Mod. with Met. And Trypt.	**-**	**0.10**	**-0.34**
	Correl Methionine with Trypt.	**-**	**-0.12**	**-**
	Min	**1.0**	**0.09**	**0.26**
	Max	**5.0**	**0.49**	**0.64**

Table C.2. QPM test crosses between inbreds with different origins and Tx804 and hybrid checks evaluated for endosperm modification, average methionine and tryptophan content in 2002.

Entry	Testcrosses with Tx804	End. Mod. Ratings	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
1	Pop. 65 Yellow Flint QPM-1-3/TX804	4.06	0.14	0.20
2	Pop. 65 Yellow Flint QPM-2-4/TX804	3.80	0.21	0.27
3	Pop. 65 Yellow Flint QPM-4-1/TX804	4.84	0.17	0.22
4	Pop. 65 Yellow Flint QPM-4-2/TX804	4.98	0.23	0.14
5	Pop. 66 Yellow Dent QPM-3-1/TX804	3.62	0.20	0.23
6	Pop. 66 Yellow Dent QPM-4-2/TX804	3.78	0.19	0.24
7	Pop. 69 Templado Amarillo QPM-1-1/TX804	2.96	0.22	0.23
8	Pop. 69 Templado Amarillo QPM-1-6/TX804	3.14	0.21	0.26
9	Pop. 69 Templado Amarillo QPM-1-8/TX804	4.00	0.20	0.21
10	Pop. 69 Templado Amarillo QPM-2-2/TX804	4.04	0.19	0.25
11	Pop. 69 Templado Amarillo QPM-2-7/TX804	3.58	0.21	0.23
12	Pop. 69 Templado Amarillo QPM-2-10/TX804	3.32	0.23	0.29
13	Pop. 69 Templado Amarillo QPM-3-1/TX804	4.58	0.15	0.20
14	Pop. 69 Templado Amarillo QPM-3-5/TX804	4.52	0.19	0.21
15	Pop. 69 Templado Amarillo QPM-3-6/TX804	3.22	0.19	0.21
16	Pop. 69 Templado Amarillo QPM-3-7/TX804	4.74	0.22	0.25
17	Pop. 69 Templado Amarillo QPM-3-8/TX804	4.42	0.19	0.21
18	Pop. 69 Templado Amarillo QPM-3-9/TX804	4.56	0.19	0.27
19	Pop. 69 Templado Amarillo QPM-3-10/TX804	4.44	0.26	0.29
20	Pop. 69 Templado Amarillo QPM-4-2/TX804	4.72	0.22	0.26
21	Pop. 69 Templado Amarillo QPM-4-4/TX804	4.68	0.18	0.22
22	Pop. 69 Templado Amarillo QPM-4-7/TX804	4.44	0.25	0.27
23	Pop. 69 Templado Amarillo QPM-4-9/TX804	3.92	0.23	0.23
24	Pop. 69 Templado Amarillo QPM-4-11/TX804	4.28	0.22	0.25
25	Pop. 69 Templado Amarillo QPM-5-4/TX804	4.58	0.19	0.26
26	Pop. 69 Templado Amarillo QPM-5-5/TX804	4.50	0.18	0.22
27	Pop. 69 Templado Amarillo QPM-5-6/TX804	3.26	0.17	0.20
28	Pop. 69 Templado Amarillo QPM-5-7/TX804	4.42	0.19	0.27
29	Pop. 69 Templado Amarillo QPM-5-12/TX804	4.28	0.24	0.26
30	Pop. 69 Templado Amarillo QPM-5-13/TX804	3.84	0.27	0.32
31	Pop. 69 Templado Amarillo QPM-6-8/TX804	3.64	0.22	0.27
32	Pop. 70 Templado Amarillo Dentado QPM-2-6/TX804	4.70	0.19	0.30
33	Pop. 70 Templado Amarillo Dentado QPM-2-7/TX804	4.70	0.19	0.25
34	Pop. 70 Templado Amarillo Dentado QPM-2-8/TX804	4.52	0.16	0.16
35	Pop. 70 Templado Amarillo Dentado QPM-2-10/TX804	4.04	0.22	0.27
36	Pop. 70 Templado Amarillo Dentado QPM-3-2/TX804	4.08	0.18	0.24
37	Pop. 70 Templado Amarillo Dentado QPM-3-3/TX804	4.42	0.21	0.22
38	Pop. 70 Templado Amarillo Dentado QPM-3-4/TX804	3.70	0.19	0.28

Table C.2. Continued.

Entry	Testcrosses with Tx804	End. Mod. Ratings	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
39	Pop. 70 Templado Amarillo Dentado QPM-3-5/TX804	4.14	0.18	0.21
40	Pop. 70 Templado Amarillo Dentado QPM-3-7/TX804	3.50	0.17	0.30
41	Pool 26 Tropical Late Yellow Dent QPM-2-1/TX804	4.30	0.16	0.21
42	Pool 26 Tropical Late Yellow Dent QPM-2-2/TX804	4.34	0.20	0.21
43	Pool 26 Tropical Late Yellow Dent QPM-3-1/TX804	2.88	0.17	0.25
44	Pool 26 Tropical Late Yellow Dent QPM-8-2/TX804	3.30	0.19	0.29
45	Pool 33 Subtropical Inter. Yellow Flint QPM-2/TX804	4.82	0.22	0.27
46	Pool 33 Subtropical Inter. Yellow Flint QPM1-4/TX804	4.68	0.15	0.20
47	Pool 33 Subtropical Inter. Yellow Flint QPM2-1/TX804	4.84	0.16	0.23
48	Pool 34 Subtropical Inter. Yellow Dent QPM-1-1/TX804	1.42	0.23	0.27
49	Pool 34 Subtropical Inter. Yellow Dent QPM-1-2/TX804	2.44	0.18	0.23
50	Pool 34 Subtropical Inter. Yellow Dent QPM-1-3/TX804	2.86	0.16	0.20
51	Pool 34 Subtropical Inter. Yellow Dent QPM-2-1/TX804	4.60	0.21	0.26
52	Pool 34 Subtropical Inter. Yellow Dent QPM-7-1/TX804	2.68	0.22	0.26
53	Temperate x Tropical High-Oil QPM-3-1/TX804	3.38	0.16	0.25
54	Temperate x Tropical High-Oil QPM-5-1/TX804	3.58	0.26	0.38
55	Temperate x Tropical High-Oil QPM-5-3/TX804	3.68	0.15	0.25
56	Temperate x Tropical High-Oil QPM-5-4/TX804	3.52	0.20	0.29
57	Temperate x Tropical High-Oil QPM-6-3/TX804	2.32	0.26	0.28
58	Temperate x Tropical High-Oil QPM-6-8 Floury/TX804	3.08	0.20	0.25
59	Temperate x Tropical High-Oil QPM-6-9 Floury/TX804	2.18	0.19	0.34
60	Temperate x Tropical High-Oil QPM-7-4/TX804	2.96	0.16	0.30
61	Temperate x Tropical High-Oil QPM-7-5/TX804	2.74	0.15	0.24
62	Temperate x Tropical High-Oil QPM-7-9/TX804	2.78	0.19	0.27
63	Temperate x Tropical High-Oil QPM-8-1/TX804	3.24	0.25	0.26
64	Temperate x Tropical High-Oil QPM-8-2/TX804	3.74	0.19	0.23
65	Temperate x Tropical High-Oil QPM-8-4/TX804	3.52	0.13	0.21
66	Temperate x Tropical High-Oil QPM-8-5/TX804	3.64	0.17	0.24
67	Temperate x Tropical High-Oil QPM-8-6/TX804	3.12	0.17	0.26
68	Temperate x Tropical High-Oil QPM-8-7/TX804	4.12	0.17	0.35
69	Temperate x Tropical High-Oil QPM-8-8/TX804	2.88	0.19	0.28
70	Temperate x Tropical High-Oil QPM-8-9/TX804	4.02	0.10	0.18
71	Tx802/TX804	3.62	0.15	0.22
72	(Tx802 x Ko326y)-18-1-1-1-/TX804	3.90	0.24	0.26
73	(Ko326y x Tx806)-6-1-1-1-/TX804	4.24	0.20	0.22
74	B73o2o2/TX804	2.14	0.17	0.16
75	Do940y/TX804	3.52	0.20	0.25
76	CML161/TX804	3.86	0.20	0.27
77	Do940y/Tx802/TX804	3.32	0.18	0.23
78	Do940y/Tx802/TX804	2.86	0.18	0.23
79	CML161/(Tx802 x Ko326y)-18-1-1-1/TX804	4.48	0.15	0.20

Table C.2. Continued.

Entry	Testcrosses with Tx804	End. Mod. Ratings	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
80	CML161/(Tx806 x Bo395y)-5-1-1-1-1/TX804	3.92	0.19	0.19
81	SL53	1.82	0.18	0.22
82	P31B13	5.00	0.24	0.16
83	P32R25	5.00	0.28	0.12
84	RX897	4.56	0.22	0.11
85	DK668	5.00	0.23	0.14
86	DK687	5.00	0.32	0.11
	Mean	**3.82**	**0.20**	**0.24**
	Std Dev	**0.81**	**0.04**	**0.05**
	Correl		**0.15**	**-0.33**
	Min	**1.42**	**0.10**	**0.11**
	Max	**5.00**	**0.32**	**0.38**

Table C.3. Opaque-2 (o2o2) and QPM inbreds (inbreds 2) evaluated for endosperm modification, average methionine and tryptophan contents in 2002.

Entry	Pedigree **of opaque-2 and QPM lines**	End. Mod. Rating	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
1	CML161/Do940y	5.0	0.21	0.27
2	CML161/Tx802	5.0	0.27	0.32
3	(CML161/(Ko326y x Tx806)-2-2-1-1-)B	5.0	0.24	0.29
4	(CML161/(Tx806 x Bo395y)-5-1-1-1-1)	5.0	0.25	0.27
5	(SH-OILQc15HC51-2-3-1-1 X CML 193)	4.7	0.28	0.30
6	Tx802 /CML161-3	4.8	0.25	0.29
7	((Tx802/CML161)x(Do940/CML161))-3	5.0	0.20	0.24
8	((Tx802/CML161)x(Do940/CML161))-4	5.0	0.23	0.23
9	(Tx802 x Ko326y)-18-1-1-1/CML161-3	4.7	0.26	0.26
10	(Tx802 x Ko326y)-18-1-1-1/CML161-4	5.0	0.25	0.29
11	((Tx802xKo326y)-18-1-1-1/CML161)x(Tx802/CML161))-1	5.0	0.19	0.20
12	((Tx802xKo326y)-18-1-1-1/CML161)x(Tx802/CML161))-2	4.4	0.25	0.36
13	(Ko326y x Tx806)-2-2-1-1/CML161-1	5.0	0.20	0.29
14	((Ko326y x Tx806)-6-1-1-1/CML161)x(Tx802/CML161))-2	4.3	0.19	0.28
15	((Tx802 x Ko326y)-18-1-1-1/CML193)x((Tx802 x Ko326y)-18-1-1-1/CML161))-4	5.0	0.26	0.27
16	(G26Qc18MH134-4-3-#-#-#-#-2 X CML 161)-1	5.0	0.32	0.31
17	(G26Qc18MH134-4-3-#-#-#-#-2 X CML 161)-4	5.0	0.28	0.27
18	(P69Qc3HC107-1-1#-4-2#-4-1-4 X CML 193)-2	5.0	0.26	0.34
19	((Tx808 x Ko326y)-6-1-1-1/Do940y)-1	4.5	0.24	0.27
20	((Tx808 x Ko326y)-6-1-1-1/Do940y)-2	4.6	0.26	0.29
21	Temp. SSEarly (B14,A632,A635,B73) B-22	1.3	0.48	0.34
22	Temp. SSLate (B37,B73,B84) B-24-1	2.7	0.45	0.33

Table C.3. Continued.

Entry	Pedigree of opaque-2 and QPM lines	End. Mod. Rating	Average Methionine	Average Tryptophan
		scale 1(op)-5(mod)	relative	relative
23	Temp. SSLate (B37,B73,B84) B-32-3	1.8	0.43	0.32
24	Temp. SSLate (B37,B73,B84) B-38	2.0	0.50	0.35
25	Temp. SSLate (B37,B73,B84) B-54-1 (1 ear)	1.3	0.43	0.36
26	Temp. SSLate (B37,B73,B84) B-55-2	1.3	0.40	0.39
27	Temp. SSLate (B37,B73,B84) B-62	2.2	0.41	0.30
28	Temp. SSLate (B37,B73,B84) B-67	2.4	0.37	0.37
29	Temp. SSLate (B37,B73,B84) B-68	1.3	0.45	0.42
30	Temp. SSLate (B37,B73,B84) B-71-2	5.0	0.37	0.33
31	Temp. SSLate (B37,B73,B84) B-76-2	2.9	0.35	0.30
32	Temp. SSLate (B37,B73,B84) B-81-1	1.2	0.30	0.32
33	Temp. SSLate (B37,B73,B84) B-81-2	4.0	0.33	0.31
34	Temp. SSLate (B37,B73,B84) B-82-2	2.4	0.33	0.34
35	Temp. SSLate (B37,B73,B84) B-86	4.5	0.30	0.26
36	Temp. SSLate (B37,B73,B84) B-89-2	4.4	0.43	0.28
37	Temp. SSLate (B37,B73,B84) B-97-2	1.0	0.32	0.32
38	Temp. SSLate (B37,B73,B84) B-103	3.0	0.28	0.27
39	Temp. NSSEarly B-31-1	1.1	0.25	0.29
40	Temp. NSSEarly B-71-1	1.0	0.23	0.30
41	Temp. NSSEarly B-161-2	1.2	0.25	0.29
42	Temp. NSSEarly B-189-2	3.8	0.36	0.34
43	Temp. NSSEarly B-196-1	3.5	0.60	0.28
44	Temp. NSSEarly B-196-3	3.2	0.45	0.29
45	Temp. NSSLate B-33	1.5	0.29	0.26
46	Temp. NSSLate B-77-1	3.2	0.30	0.27
47	Temp. NSSLate B-77-2	1.2	0.22	0.32
48	Temp. NSSLate B-89-2	1.0	0.28	0.30
49	Temp. NSSLate B-103-2	1.0	0.22	0.28
50	Temp. NSSLate B-105	1.2	0.29	0.28
51	Temp. NSSLate B-117-2	4.1	0.30	0.33
52	Temp. NSSEarly B-69	1.0	0.27	0.30
53	(Tx802/B104)-1-2-1	1.0	0.34	0.36
54	(Tx802/B104)-1-2-3	1.5	0.42	0.37
55	(Tx802/B104)-2-51-1	5.0	0.50	0.26
56	(Tx802/B104)-3 OPAQUE-6-3	1.0	0.27	0.46
57	(Tx802/B104)-3 OPAQUE-8-2	1.0	0.23	0.30
58	(Tx802/B104)-3 OPAQUE-121-3	1.5	0.28	0.36
59	(Tx802/B104)-3 OPAQUE-122&3-2	1.0	0.20	0.28
60	((NC300/(Do940y x Tx802)-4-2-1-1-1)x(Tx770/CML161))-2	1.0	0.25	0.34
61	((Ko326y x Tx806)-6-1-1-1/NC300)x(Tx770/CML193))1	1.1	0.24	0.34
62	(B97/Tx802)x(NC300/(Do940y x Tx802)-4-2-1-1-1)--2	5.0	0.31	0.22
63	(B97/Tx802)x(NC300/Do940y)1	1.6	0.22	0.26
64	(B97/Tx802)x(NC300/CML161)-2	1.7	0.21	0.27
65	(B97/Do940y))-1	1.4	0.17	0.25
66	(B97/Do940y)x(NC300/CML161)2-2	2.0	0.19	0.26

Table C.3. Continued.

Entry	Pedigree of opaque-2 and QPM lines	End. Mod. Rating scale 1(op)-5(mod)	Average Methionine relative	Average Tryptophan relative
67	(B97/(Ko326y x Tx806)-6-1-1-1)x(NC300/Tx802)	4.7	0.31	0.23
68	(B97/(Ko326y x Tx806)-6-1-1-1)x(NC300/Tx802)-3	1.3	0.22	0.29
69	(B97/(Ko326y x Tx806)-6-1-1-1)x((Ko326y x Tx806)-6-1-1-1/NC300)13	1.3	0.21	0.29
70	(B97/(Ko326y x Tx806)-6-1-1-1)x(NC300/CML161)-1	1.0	0.24	0.28
71	(B97/(Ko326y x Tx806)-6-1-1-1)x(NC300/CML161)-2	1.0	0.22	0.34
72	((B104/B73 o2o2)x(B104/Tx802))2-2	1.0	0.23	0.33
73	((B104/B73 o2o2)x(Tx714/CML193))(Tx714/CML193))-1	1.0	0.21	0.28
74	((B73 o2/o2 /B104)x(Tx714/(Ko326y x Tx806)-6-1-1-1))-2	1.0	0.30	0.33
75	(B104-1/(Tx802 x Ko326y)-18-1-1-1))-2	1.5	0.30	0.37
76	(B104-1/(Tx802 x Ko326y)-18-1-1-1))-3	1.0	0.27	0.30
77	(B104-1/(Tx802 x Ko326y)-18-1-1-1))-4	1.1	0.28	0.31
78	((B104/(Tx802 x Ko326y)-18-1-1-1)x(Tx714/(Ko326y x Tx806)-6-1-1-1))1	1.1	0.30	0.41
79	((B104/(Tx802 x Ko326y)-18-1-1-1)x(Tx714/(Ko326y x Tx806)-6-1-1-1))2	3.4	0.26	0.28
80	((B104-1/CML 193)x(Tx714/(Ko326y x Tx806)-6-1-1-1))	1.0	0.29	0.36
81	((Ko326y x Tx806)-6-1-1-1/B104))	1.0	0.29	0.38
82	((Ko326y x Tx806)-6-1-1-1/B104))	1.0	0.25	0.32
83	((Tx770/CML161)x(B97/Do940y)-1	1.0	0.24	0.35
84	B73 o2	1.2	0.33	0.42
85	CML161	5.0	0.22	0.22
86	FRB73	1.5	0.49	0.23
	Mean	**2.6**	**0.30**	**0.31**
	Std Dev	**1.7**	**0.09**	**0.05**
	Correl. End. Mod. with Met. And Trypt.	**-**	**-0.02**	**-0.46**
	Min	**1.0**	**0.17**	**0.20**
	Max	**5.0**	**0.60**	**0.46**

APPENDIX D

Table D.1. Pedigree of 92 high lysine maize inbred lines used in diversity analysis of chromosome 7.

Entry	Code	Pedigree
1	802(a)	Tx802
2	804	Tx804
3	b/a(g)	(Do940y/Tx802)-4-2-1-1-1
4	a/Ko(e)	(Tx802/Ko326y)-18-1-1-1
5	Ko/d(f)	(Ko326y/Tx806)-6-1-1-1
6	Do(b)	Do940y
7	161(c)	CML161
8	806(d)	Tx806
9	B73o2(L)	B73 o2
10	T220o2	T220 o2
11	a/c	(Tx802-B /CML161)-B-3-B
12	e/c	((Tx802/Ko326y)-18-1-1-1/CML161)-B-4-B
13	Ko/d/c	((Ko326y/Tx806)-2-2-1-1/CML161)-B-1-B
14	f/c/e	((Ko326y/Tx806)-6-1-1-1/CML161)/(Tx802/CML161))-1-B
15	e/e/c	((Tx802/Ko326y)-18-1-1-1/CML193)/((Tx802 x Ko326y)-18-1-1-1/CML161))-4
16	G26cB1	(G26Qc18MH134-4-3-#-#-#-#-2/CML 161)-1-B
17	G26cB4	(G26Qc18MH134-4-3-#-#-#-#-2/CML 161)-4-B
18	P69Q/193	(P69Qc3HC107-1-1#-4-2#-4-1-4-B/CML 193)-2-B
19	P65	Pop. 65 Yellow Flint QPM-B4-2-B
20	P69B16	Pop. 69 Templado Amarillo QPM-B1-6-B
21	P69B18	Pop. 69 Templado Amarillo QPM-B1-8-B
22	P69B22	Pop. 69 Templado Amarillo QPM-B2-2-B
23	P69B212	Pop. 69 Templado Amarillo QPM-B2-12-B
24	P69B35	Pop. 69 Templado Amarillo QPM-B3-5-B
25	P69B47	Pop. 69 Templado Amarillo QPM-B4-7-B
26	P69B52	Pop. 69 Templado Amarillo QPM-B5-2-B
27	P70	Pop. 70 Templado Amarillo Dentado QPM-B2-8-B
28	HighOil	Temperate x Tropical High-Oil QPM-B-7-B-9-B
29	NC/gc(h)	((NC300/(Do940y/Tx802)-4-2-1-1-1)/(Tx770-B/CML161))-2
30	B97(J)ah	(B97/Tx802)/(NC300/(Do940y/Tx802)-4-2-1-1-1)-2
31	Ja/NCc	(B97/Tx802)/(NC300/CML161)-1
32	Ja770c	(B97/Tx802)/(Tx770-B/CML161)-1
33	JdBoNCMo	(B97/(Tx806/Bo395y)-5-1-1-1-1)/(NC300/Mo17 o2)-2
34	JdBoNCh	(B97/(Tx806/Bo395y)-5-1-1-1-1)/(NC300/(Do940y/Tx802)-4-2-1-1-1)-2
35	J/b	(B97/Do940y))-B-1
36	J/bNC/c	(B97/Do940y)/(NC300/CML161)-B2-B-2
37	J/f/NCa1	(B97/(Ko326y/Tx806)-6-1-1-1)/(NC300/Tx802)-1
38	J/f/NCa3	(B97/(Ko326y/Tx806)-6-1-1-1)/(NC300/Tx802)-3
39	J/f/f/NC	(B97/(Ko326y/Tx806)-6-1-1-1)/((Ko326y x Tx806)-6-1-1-1/NC300)-B1-B-2
40	J/f/NC/c	(B97/(Ko326y/Tx806)-6-1-1-1)/(NC300/CML161)-2
41	K/L/K/a	((B104/B73 o2o2)/(B104/Tx802))-B2-B-2
42	K/e	((B104-1/(Tx802/Ko326y)-18-1-1-1))-B-3
43	K/e714/f	((B104/(Tx802/Ko326y)-18-1-1-1)/(Tx714/(Ko326y/Tx806)-6-1-1-1))-2
44	f/104(K)	((Ko326y/Tx806)-6-1-1-1/B104))-B
45	a/K18	(Tx802-B/B104)-1-18-B-1
46	a/K25	(Tx802-B/B104)-2-5-B1-1
47	a/Ko21	(Tx802-B/B104)-3 OPAQUE-2-B-1
48	a/Ko212	(Tx802-B/B104)-3 OPAQUE-12-B2&3-2

Table D.1. Continued.

Entry	Code	Pedigree
49	SSE19	Temp. SSEarly (B14,A632,A635,B73) B-19-B
50	SSE22	Temp. SSEarly (B14,A632,A635,B73) B-22-B
51	SSL5	Temp. SSLate (B37,B73,B84) B-5-B-2
52	SSL12	Temp. SSLate (B37,B73,B84) B-12-B-2
53	SSL18	Temp. SSLate (B37,B73,B84) B-18-B
54	SSL22	Temp. SSLate (B37,B73,B84) B-22-B
55	SSL24	Temp. SSLate (B37,B73,B84) B-24-B-1
56	SSL29	Temp. SSLate (B37,B73,B84) B-29-B
57	SSL38	Temp. SSLate (B37,B73,B84) B-38-B
58	SSL39	Temp. SSLate (B37,B73,B84) B-39-B
59	SSL40	Temp. SSLate (B37,B73,B84) B-40-B
60	SSL44	Temp. SSLate (B37,B73,B84) B-44-B
61	SSL46	Temp. SSLate (B37,B73,B84) B-46-B-3
62	SSL61	Temp. SSLate (B37,B73,B84) B-61-B-2
63	SSL62	Temp. SSLate (B37,B73,B84) B-62-B
64	SSL64	Temp. SSLate (B37,B73,B84) B-64-B-3 (1 ear)
65	SSL66	Temp. SSLate (B37,B73,B84) B-66-B
66	SSL68	Temp. SSLate (B37,B73,B84) B-68-B
67	SSL71B1	Temp. SSLate (B37,B73,B84) B-71-B-1
68	SSL71B2	Temp. SSLate (B37,B73,B84) B-71-B-2
69	SSL76	Temp. SSLate (B37,B73,B84) B-76-B-2
70	SSL81	Temp. SSLate (B37,B73,B84) B-81-B-2
71	SSL89	Temp. SSLate (B37,B73,B84) B-89-B-1
72	SSL103	Temp. SSLate (B37,B73,B84) B-103-B
73	SSL108	Temp. SSLate (B37,B73,B84) B-108-B
74	NSE7	Temp. NSSEarly B-7-B-1
75	NSE31	Temp. NSSEarly B-31-B-1
76	NSE71B1	Temp. NSSEarly B-71-B-1
77	NSE123	Temp. NSSEarly B-123-B-1
78	NSE125	Temp. NSSEarly B-125-B-2
79	NSE161B1	Temp. NSSEarly B-161-B-1 (1 ear)
80	NSE161B2	Temp. NSSEarly B-161-B-2
81	NSE177B2	Temp. NSSEarly B-177-B-2
82	NSE196B1	Temp. NSSEarly B-196-B-1
83	NSE196B3	Temp. NSSEarly B-196-B-3
84	NSL45	Temp. NSSLate B-45-B
85	NSL71	Temp. NSSLate B-71-B
86	NSL771	Temp. NSSLate B-77-1
87	NSL772	Temp. NSSLate B-77-2
88	NSL87	Temp. NSSLate B-87-B-1
89	NSL89	Temp. NSSLate B-89-B-1
90	NSL89	Temp. NSSLate B-89-B-2
91	NSL103	Temp. NSSLate B-103-B-2
92	NSL105	Temp. NSSLate B-105-B

Legend: B = Bulk of selfs, SS = Stiff Stalk, NSS = Non Stiff Stalk.

Table D.2. Bin no. of single sequence repeats, repeat sequence, primer sequence, annealing temperature and expected allele size in base pairs used in diversity analysis of chromosome 7 in maize.

Bin no.	SSR	Repeat Sequence	Primer Sequence	Annealing Temp.	Allele Size
7.0055	umc1545	(AAGA)4	GAAAACTGCATCAACAACAAGCTG // ATTGGTTGGTTCTTGCTTCCATTA	55	80
7.0138	umc1241	(GTCTTTG)4	TGAAGCAAGTCACTGGTAAGAGCA // TGACACACCCATACTTCCAACAAG	55	159
7.02	umc1480	(GAA)4	AATGAAGGTGGATGTGCTGCTACT // CTTCCCCATCTCCTCTTGAAGATT	55	144
7.0478	umc1426	(AGAGG)4	TAGGGTCGATTCTGGATTGTCTG // TGTAAAACAGAAAGCATGCGAGTC	54	136
7.0533	bnlg2132	(AG)21	GGCGAGAGAGGCAAAGTTAA // GTCGCACAAGGGGATCAC	51	200-250
7.07952	bnlg1292	(AG)14	GGCGCGCACATAGCTC // GCCTGGGCTGGCTTCA	52	200-250
7.092	umc1159	(AG)8	TTCCCATGTTCATTTCAGGTTCTT// TCATGGGTTTTGAGGCTGTATTTT	53	144
7.10765	umc2364	(GGA)7	AACCTCAAGATCACCAACATCCTC// CACCCTGCTGTCAGATGGATACTT	57	-
7.11468	umc2392	(GGC)5	CAGAGACCTCGACTTCGACCAC//CTTCT GCTTCTGCTCGACCTTCT	58	142
7.1185	umc2160	(AG)10	TAAAACCTTTACCCCATCCAGCAT//TGTG CTCGTGCTTCTCTCTGAGTA	57	160
7.1224	phi057	(GCC)4	CTCATCAGTGCCGTCGTCCAT // CAGTCGCAAGAAACCGTTGCC	58	154
7.12245	umc1066	(GCCAGA)5	ATGGAGCACGTCATCTCAATGG // AGCAGCAGCAACGTCTATGACACT	56	156
7.1252	umc1577	(CTTGGC)4	TTTCCCTTCTTGGCAGGAGC // AAGAACTCCTTCAAGCTGCCG	55	296
7.12782	bnlg1367	AG(42)	CGACGGCGTACAGAGAGAG // GGTCGCCACCCCACCT	52	125-140
7.1286	bnlg2160	AG(27)	GAAGCAACCCATTTTCATCC // AGATTGGATTCCTGCCTCCT	51	110-150
7.132	umc1068	(GAAAA)6(GAA)2	AGTCGTTTTCAAAGGCTGCTGATA // TGAGTCACCTCATTTCTTCTGGTTC	55	136
7.14243	bnlg1200	(AG)24	CGTCCTCGTTGTTATTCCGT // GTTCCCTCTCTCCCTCCCTC	52	226
7.153	umc1401	(CCA)4	CTCTGGTCCATCCTCATCGACT // TCTCTTGATCACATATCGATCCCA	54	151
7.1558	umc1016	(CT)25	GTGATACCGGGTAATCTGGTGC // GATGATGGGTGATCATCGGTTC	55	129
7.158	umc2327	(TCTC)5	GATCGATGCTAATGTGAAGAGCCT // CCAGCAGCATATGTACACAAATCA	54	76-97
7.1799	phi034	(CCT)4	TAGCGACAGGATGGCCTCTTCT // GGGGAGCACGCCTTCGTTCT	58	120-141
7.2	umc2098	(CAG)5	GGTGAACAAGATCTCTTTGTCTACTGT // CCTTCTCGGCCATTATTGCT	53	93
7.2463	umc2142	(AGG)4	ATGGATCAGGGGAAAGAGCAA // CCTCCTCGTCCTCCTTCTTGAT	55	124
7.2477	umc1138	(AC)6	ATCAGCATCCTCCATTCACACAT // CGGGAAATGCTAGAATTATGCTGA	55	110
7.2524	umc1787	(CGG)4	TGTAGTCCATGGAGCTCTTCTCCT // CTTTTTCACACTCTGCACCTCCTC	55	86
7.26355	umc1585	(TGG)7	CGGCCTATGTAACAATCCCTAGC // AAGGGAAAGAATAATCCAACCGTC	55	137
7.2927	mmc0411	(CT)29	CGATGCAAGAGTGTCAAGTA // ACTCCCTAGTGCAAAAATCA	46	152-181
7.3084	umc1567	(AGA)4	GCGGCAGGAGTACTCACTATATGC // GTCCGAGAATAAGATCGTTGATGG	55	142

Table D.2. Continued.

Bin no.	SSR	Repeat Sequence	Primer Sequence	Annealing Temp.	Allele Size
7.33424	umc1456	(AACC)5	GCCACAGCTCACTAGCTCAAAAGT // CTCTGTGTGTTTGCTTGATTGCTT	55	142
7.3654	umc1408	(CGG)5	GATCCGTCTCTTGCCGTGGTA // ATGAGCTTGCGGTCCTCCTC	56	160
7.37161	phi114	(GCCT)3	CCGAGACCGTCAAGACCATCAA // AGCTCCAAACGATTCTGAACTCGC	58	135-166
7.3812	umc1134	(AGC)7	AAAACTAACAGGCAGCAGACCAAC // ATCAGCAAGTGACTGAATTCCTCC	55	86
7.3921	bnlg1805	AG(29)	GCCCGTTTGCTAAGAGAATG // TGTTCGAGCATTTGCTCTTG	51	275-300
7.4105	umc1710	(CTG)5	ACTTTGCAACTACCGTACATGGGT // TTCGACTGCACGTGAAAATCTATC	55	93
7.4305	bnlg1666	(AG)34	GCTGGTAGCTTTCAGATGGC // TGTCCCTCCTCCAGTTTCAC	51	100-150
7.45391	umc1782	(GAC)4	CGTCAACTACCTGGCGAAGAA // TCGCATACCATGATCACTAGCTTC	54	135
7.4729	phi328175	AGG	GGGAAGTGCTCCTTGCAG // CGGTAGGTGAACGCGGTA	51	101-130
7.4892	bnlg2259	(AG)17	ACCATTGATTTCATGGTATTGG // GCGGATAATGACATTGGGTC	51	160-180
7.51741	bnlg2328	(AG)33	AGCAGTGAGGAAGAAGCAGG // TTACCCTCCCTTGTCGTGAC	51	110-160
7.5452	phi069	GAC	AGACACCGCCGTGGTCGTC // AGTCCGGCTCCACCTCCTTC	57	197-206
7.5989	umc1406	(CTCA)4	AGAGGAGACAGGAGGTCGGTAGTT // TGTGGTGTGGTCTTCTCTCTTCTG	55	106
7.6115	phi116	ACTG/ACG***	GCATACGGCCATGGATGGGA // TCCCTGCCGGGACTCCTG	59	154-173
7.64414	phi051	AGG	GGCGAAAGCGAACGACAACAATCTT // CGACATCGTCAGATTATATTGCAGACCA	61	140-150

List of genetic analysis software.

1. *Arlequin* -- http://anthropologie.unige.ch/arlequin/ see Excoffier
2. Assignment tests -- Paetkau/Brzustowski http://www.biology.ualberta.ca/jbrzusto/Doh.html Based on Paetkau et al. 1995. Mol. Ecol. 4: 347 (Msat refs.doc) Cornuet, J.-M. http://www.ensam.inra.fr/campus/index-recherche.html
3. *BLAST* -- NIH site for finding related DNA sequences http://www.ncbi.nlm.nih.gov/BLAST/
4. *Bottleneck* -- -- Cornuet, J.-M. http://www.ensam.inra.fr/campus/index-recherche.html
5. *Cervus* -- Marshall, T. http://helios.bto.ed.ac.uk/evolgen/cervus/cervusregister.html
6. *ClustalX* -- sequence alignment software http://innprot.weizmann.ac.il/software/ClustalX.html

7. *Excel Microsatellite Toolkit* Park, Stephen. Smurfit Institute of Genetics, Trinity College, Dublin 2,Ireland spark@tcd.ie / sdepark@hotmail.com; Tel: +353 (0)1 608 3538/1265http://acer.gen.tcd.ie/~sdepark/ms-toolkit/

8. *FSTAT* -- Goudet: (see also Raymond & Rousset GENEPOP) http://www.unil.ch/izea/softwares/fstat.html see Goudet

9. *GDA* -- Lewis, P.O. http://lewis.eeb.uconn.edu/lewishome/software.html

10. *GENECLASS* -- Cornuet, J.-M. http://www.ensam.inra.fr/campus/index-recherche.html

11. *GENEPOP* -- Raymond M. & Rousset F, 1995. GENEPOP (version 3.3): population genetics software for exact tests and ecumenicism. J. Heredity, 86:248-249ftp://ftp.cefe.cnrs-mop.fr/pub/pc/msdos/genepop

12. *GeneStat* Lewis, P.O. http://lewis.eeb.uconn.edu/lewishome/software.html

13. Goudet: http://www.unil.ch/izea/softwares/fstat.html

14. *PHYLIP* – Felsenstein http://evolution.genetics.washington.edu/

15. *PowerSSR* - Liu, J. http://www.stat.ncsu.edu/~kliu2/index.htm

16. *Structure* - Pritchard, J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959. http://pritch.bsd.uchicago.edu/

17. *TFPGA* Miller, Mark P. Tools for Population Genetic Analyses TFPGA ASU post-doc
   Mark.Miller@cnr.usu.edu http://bioweb.usu.edu/mpmbio/

18. *TreeView* -- Page, Rod. http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

19. *WINAMOVA* -- Michalakis & Excoffier http://acasun1.unige.ch/LGB/Software/Windoze/amova

Measures produced:

Alignment of DNA sequences:	*ClustalX*
Cavalli-Sforza distances:	*PHYLIP, TFPGA* (?)
F-statistics:	*FSTAT, GDA, GenePop, GeneStat, Genetix.*
Gene diversity (D):	*GeneStat, TFPGA, Genetix.*
Gene frequencies:	(from genotypic data) *FSTAT, Relatedness*, others.
G_{ST}:	*GeneStat, FSTAT, TFPGA.*
Hardy-Weinberg fit:	*GenePop, FSTAT, TFPGA, Arlequin.*
Mantel tests:	*Genetix, TFPGA, McMantell.*
N_e (effective pop. size):	*Migrate, Misat.*
Nei's distance('72, '78):	*GeneStat, GDA, FSTAT, TFPGA (PHYLIP* Nei's 1972 only*).*
Nested clade	*GeoDis*
PCA:	Principal comp. Analysis w/ *PCP, PCAGen, MiniTab*
Relatedness	*Relatedness, Identix, SPAGeDi*
R_{ST}:	*FSTAT, Genetix, R_{ST}Calc.*
Rogers' distance:	*TFPGA, GeneStat.*
Theta (Θ):	*GDA, FSTAT.* (Cockerham & Weir *F*-stat)
Tree diagrams:	*TreeView*
Θ:	PowerSSR, *GDA, FSTAT, Genetix.* (Cockerham & Weir *F*-stat)

VITA

Sandeep Bhatnagar was born on July 12, 1965 to Dr. Aditya Nath Bhatnagar and Mrs. Kusum Bhatnagar in the beautiful city of Dehradun, Uttar Pradesh, India. He completed his B.S. in agriculture from the University of Allahabad, U.P., India in 1987 and M.S. in plant breeding from G. B. Pant University of Agriculture and Technology, Pantnagar, U.P., India in April 1990.

In the fall 1999, he enrolled at Texas A&M University in the maize breeding and genetics program to pursue another M.S. in plant breeding, which he completed in August 2001 and then continued to complete his Ph.D. in December, 2004.

He is married to Mrs. Sandhya Bhatnagar and has three children, Samarthya, Shriya and Sanjula.

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