MAHARANA PRATAP UNIVERSITY OF AGRICULTURE & TECHNOLOGY, UDAIPUR

RAJASTHAN COLLEGE OF AGRICULTURE, UDAIPUR

CERTIFICATE-I

Dated / /2014

This is to certify that **Mr. Radheshyam Sharma** has successfully completed the comprehensive examination held on 16th July, 2012 as required by the regulation for degree of **Doctorate of Philosophy in Agriculture (Molecular Biology and Biotechnology).**

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CERTIFICATE-II

Dated: / / 2014

This is to certify that the thesis entitled **"Assessment of Morphological, Biochemical and Molecular Diversity in *Zea mays* L."** submitted for the degree of **Doctorate of Philosophy** in the subject of **Molecular Biology and Biotechnology,** embodies bonafied research work carried out by **Mr. Radheshyam Sharma** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of this thesis was also approved by the advisory committee on ----------.

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CERTIFICATE-III

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This is to certify that the thesis entitled **"Assessment of Morphological, Biochemical and Molecular Diversity in *Zea mays* L."** submitted by **Mr. Radheshyam Sharma** to the Maharana Pratap University of Agriculture and Technology, Udaipur in partial fulfillment of the requirements for the degree of **Doctorate of Philosophy** in the subject of **Molecular Biology and Biotechnology** after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination on her thesis has been found satisfactory; we therefore, recommended that the thesis should be approved.

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CERTIFICATE-IV

Dated: / /2014

This is to certify that **M**r. **Radheshyam Sharma** of the Department of **Molecular Biology and Biotechnology,** Rajasthan College of Agriculture, Udaipur has made all corrections and modifications in the thesis entitled **"Assessment of Morphological, Biochemical and Molecular Diversity in *Zea mays* L."** which were suggested by the external examiner and the advisory committee in the oral examination held on / /2013. The final copies of the thesis duly bound and corrected were submitted on / /2013 are enclosed here with for approval.

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Enclosed one original and two copies of bound thesis forwarded to the Director, Resident Instruction, MPUAT, Udaipur, through the Dean, Rajasthan College of Agriculture, Udaipur.

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Assessment of Morphological, Biochemical and Molecular Diversity in *Zea mays* L.

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ABSTRACT

The present investigation was carried out with 20 diverse genotypes of Maize (*Zea mays* L.) comprising hybrids, composites and land races for the assessment of genetic diversity through morphological, biochemical (Isozyme) and molecular (RAPD and ISSR) markers. The crop was raised during *kharif*, 2011 in randomized block design with three replications at Instructional Farm of Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India. Observations were recorded for all 11 characters including grain protein content.

Data so obtained were subjected to analysis of variance, genotypic variability parameters, correlation, path coefficient analysis and genetic divergence analysis. For biochemical characterization enzymes were extracted from young leaves (28 DAS) to develop zymograms. Similarly for molecular analysis, DNA was isolated in seedling stage (21-25 DAS) and used as a template for PCR amplification using 16 RAPD and 10 ISSR primers.

Analysis of variance revealed significant genetic variability among the genotypes for all the 11 characters including grain protein content. Test weight, grain yield per plant, grains per cob, grains per row and harvest index revealed high genetic gain along with high estimate of heritability and GCV indicating that selection could be practiced for improvement. Correlation coefficient revealed close association at genotypic and phenotypic level. Grain yield per plant was strongly correlated with days of 50% flowering, days of maturity, cob length, grains per cob, cob girth, grains per row and harvest index. Path coefficient analysis indicated that harvest index, biological yield and grains per row exhibited significant direct effect on seed yield in maize.

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On the basis of various variability parameters selection for harvest index, biological yield and grains per row appeared the most important contributing characters for enhancing the productivity. Genotypes HQPM-1, HQPM-5 and EQH-63 were superior for most important yield and quality characters and could be gainfully utilized in breeding programmes. Further, through genetic divergence analysis all the 20 genotypes were classified into six divergent clusters. Inter-cluster distance ranged from 22.12 to 91.55. Maximum contribution to genetic divergence was again through test weight, harvest index, grains per row and plant height.

In isozyme analysis, a total of 8 putative isozyme alleles were generated by three enzyme system *viz.* esterase, peroxidase and superoxide dismutase. Superoxide dismutase isozyme showed 63% polymorphism and rest two were monomorphic for all alleles. Jaccard's Similarity Coefficient lies from 0.88 to 1.00 and all the genotypes were mainly divided into two clusters. Genetic diversity and distance derived from isozyme analysis were very low due to the small number of polymorphic alleles.

Molecular characterization using RAPD and ISSR marker were revealed high polymorphism upto 73.41 and 86.44 respectively. RAPD and ISSR similarity matrix data revealed that the similarity indices for different genotypes ranged from 0.34 to 0.87 with an average 0.57, again indicating a very high level of genetic similarity among the genotypes. Based on dandrogram genotypes were mainly divided into two major clusters.

Further, RAPD primer OPP-05 gave bright, constant and unique band of 1.2 kb in genotype HQPM-5 which was also further sequenced at Xeleris Pvt Ltd. Ahmadabad and developed SCAR primer for further genetic improvement.

Based on morphological, biochemical and molecular analysis it was concluded that various genotype of *Z. mays* L. included in the present study showed high genetic diversity. Critical analysis of genetic variation among the genotypes revealed that molecular markers can be precisely used in overcoming the environmental factors as evident from the values so obtained in terms of variability. On the basis of *per se* performance and all other parameters, HQPM-5, HQPM-1 and EQH63 turned out to be high yielding lines with superior grain protein content. Therefore, these could be gainfully utilized in maize improvement programmes.

1. INTRODUCTION

Maize (*Zea mays* L.), is one of the important cross-pollinated crops belongs to the tribe Maydeae of the grass family Poaceae. The plant is native to South America. *Z. mays* L. is the only species in the genus *Zea* with chromosome number 2n=20 (Bremer *et al.*, 2003). Maize is widely cultivated in tropics, sub-tropics and temperate regions. The suitability of maize to diverse environments is unmatched by any other crop as it has a broad range of plasticity. Maize enjoy one of the highest yielding among world's major coarse cereal crops of its own nature, *viz*., efficient utilization of radiant energy and fixation of carbon dioxide from the atmosphere. It is the third most important crop consumed and utilized in different forms in the world after wheat and rice.

The United States produces almost half of the world's production while, other major maize producing countries are China, Brazil, Mexico, France, Indonesia and South Africa. India ranks fourth in maize production with 21.73 m t and fifth in area of 8.55 m ha with a productivity of 26.81 q/ha as compared to world's average 5.12 t/ha (Anonymous., 2012).

In India maize is known as "Queen of cereals" and is grown from latitude 58^0 N to 40^0 S from sea level to higher than 3000 m altitude and in areas receiving yearly rainfall of 250 to 5000 mm (Downsell *et al*., 1996). In India it is mainly grown in Uttar Pradesh, Bihar and Rajasthan. The productivity of maize in Rajasthan is approximately 17.46 q/ha which is less than national average. Composites occupy significant area in our country but these are poor yielders. In Rajasthan, maize is grown in arid to semi-arid condition in 9.6 lakh ha with a production of 16, 87,200 tonnes (Government of Rajasthan, 2011-12). Maize growing districts of Rajasthan are Udaipur, Chittorgarh, Bhilwara, Banswara, Rajsamand and Ajmer.

Maize has multiple uses. The kernel contains about 77 per cent starch, 2 per cent sugar, 9 per cent protein, 5 per cent oil, 5 per cent pentosan and 2 per cent ash on a water-free basis. The ash of the kernel contains salts calcium, magnesium, phosphorus, aluminium, iron, sodium, potassium and chlorine (Kadam *et al.*, 2012). Protein content in the maize genotypes varies and recently high quality maize protein varieties were developed to increase the quality and quantity maize protein.

Maize is used primarily as a food for humans in most areas of the world, whereas, in United States, about 85 per cent of the crop is used as cattle feed. Maize grain is extensively used for industrial purpose in the preparation of corn starch, corn syrup, corn oil dextrose, corn flakes, gluten, grain cake, lactic acid and acetone. With the development of poultry and livestock industry, its consumption as animal feed has also increased tremendously. It is used in the human diet in both fresh and processed forms. Maize oil has high poly unsaturated fatty acid content and low in linoleic acid (0.7%) and contains high level of natural flavor.

Knowledge about germplasm diversity and genetic relationships among breeding materials could be an invaluable aid in crop improvement strategies (Mohammadi and Prasanna, 2003). The genetic diversity is analyzed by using morphological, biochemical as well as molecular markers. Most of the conventional breeding programmes are based over evaluation and characterization through morphological traits, which are largely affected by environmental fluctuations. Similarly, biochemical traits/tools are also used which are also sensitive to the analytical procedures. However, molecular tools hold the promise of allowing the identification of genes involved in a number of traits including adaptive traits and polymorphisms causing functional genetic variation. Molecular techniques for detecting differences in the DNA of individual plants to examine variability amongst cultivars are useful for identification of potential parental lines. These differences in general are called molecular marker. DNA markers provide a direct measure of genetic diversity and go beyond diversity based on agronomic traits or geographic origin (Dreisigacker *et al*., 2005), thus help in better germplasm management and develop more efficient strategies for crop improvement.

Genetic polymorphism in maize plants has been studied which helps in distinguishing plants at inter- and/or intra-species level (Joshi *et al.,* 2004). Different molecular marker systems such as AFLP (Altintas *et al.,* 2008; Tatikonda *et al.,* 2009), ISSR (Carvalho *et al.,* 2009; Parvathaneni *et al.,* 2011), RAPD (Kumar *et al.,* 2009) and SSR (Pagnotta *et al.,* 2009; Zarkti *et al.,* 2010; Zaher *et al.,* 2011) have been used. PCR based methods including randomly amplified polymorphic DNA (RAPD) technique (Williams *et al.,* 1990; Kumar *et al.,* 2009) a most are popular because of speed, low cost and the use of minute amount of plant material for analysis. Similarly, inter simple sequence repeat (ISSR) marker is highly effective in

plant fingerprinting and phylogenetic studies (Vaillancourt *et al.,* 2008). Easy handling, reliability and high information levels are the features that justify the utility of ISSR markers in DNA fingerprinting and genetic variability analysis (Malik *et al.*, 2010). RAPD markers are sensitive to PCR conditions hence, difficult to reproduce and therefore preferentially converted to more specific sequence characterized amplified region (SCAR) markers. SCAR primers are unique, efficient co-dominant and reproducible (Hernández *et al.*, 2003).

Most maize diversity remains undescribed, poorly understood and under utilized largely because of the difficulty of identifying useful genetic variants hidden in the background of low yielding local varieties or lines (Tanksley and McCouch, 1997). Thus identification of existing diversity in maize genotype is crucial for plant breeding programme. In recent years there has been a perceptible improvement in maize production in India, however, production and productivity in India is relatively less and lot of scope is there for improvement.

Considering the importance of maize in the State's economy in terms of stable area, production, productivity and its large consumption, the present study is proposed using 20 diverse *elite* lines comprising hybrids, composites and land races to analyse genetic diversity critically at morphological, biochemical and molecular level with the following objectives:

1. Assessment of genotypic diversity through morphological and biochemical characterization of *Z. mays* L. genotypes.
2. Analysis of molecular diversity using randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers.
3. Development of sequence characterized amplified regions (SCAR) markers for identification of closely related *Z. mays* L. genotypes, and

On the basis of assessment of above parameters superior entries were identified for future maize breeding programmes.

2. REVIEW OF LITERATURE

Understanding the molecular basis of the essential biological phenomena in plants is crucial for effective conservation, management and efficient utilization of plant genetic resources. In particular, an adequate knowledge of genetic diversity is of fundamental interest in basic science research as well as applied aspects like the efficient management of crop genetic resources which is dependent on continuous infusion of wild relatives, traditional varieties and the use of modern breeding techniques. These processes require an assessment of diversity so as resistant and highly productive varieties.

The assessment of genetic diversity within and between populations is performed at molecular level using various laboratory-based techniques such as allozyme or DNA analysis, which measure levels of variation directly. Genetic diversity may also be gauged using morphological and biochemical characterization and evaluation:

i. Morphological characterization does not require expensive technology, instead large tracts of land are often required for these experiments, making it possibly more expensive than molecular assessment. These traits are often susceptible to phenotypic plasticity conversely, this allows assessment of diversity in the presence of environmental variation.

ii. Biochemical analysis is based on the separation of proteins and their characterization (specific banding patterns). It is a fast method which requires only small amounts of biological material. However, only a limited number of enzyme systems are available and the resolution of diversity is limited.

iii. Molecular analysis comprises a large variety of DNA molecular markers, which can be employed for analysis of variation with great precision. Different markers have different genetic qualities (they can be dominant or co-dominant can be amplified anonymous or characterized loci, can contain expressed or non-expressed sequences etc.).

The available literature pertinent to present investigation primarily in maize has been reviewed and presented under following heads:

1. Morphological characterization
2. Biochemical characterization

3. Molecular characterization, and
4. Development of sequence characterized amplified regions (SCARs) marker

2.1 Morphological Characterization

Crop varieties show wide fluctuations in their yielding ability when grown over varied environments or agro-climatic zones. Morphological variants within a species are the most common of epigenetic changes that are easiest to identify. Polymorphism in morphological characters is the most widely studied phenomena by ecologists as well as taxonomists. The variations usually affect plant height, branches, root morphology, multiplicity, variegation and pigmentation of leaves, scent and colour of flower, number, shape and size of leaves. Each genotype has a specific environment for its maximum performance and similarly in a specific environment, a specific genotype performs better. This approach has limited use since it is difficult to determine part of the genome that represents the characters, their genetic control being complex or unknown in most cases due to interaction with the environment (Smith and Smith, 1991). Therefore, polymorphic markers are needed for identification of varieties, for estimation of genetic similarity among and between the populations.

Maize is preferred in formal, scientific, and international usage. The maize plant is on the average 2.5 m (meters) in height. The stem has the appearance of a bamboo cane and is commonly composed of 20 internodes of 18 cm (7 inches) in length. A leaf grows from each node, which is generally 9 cm (3.5 inches) in width and 120 cm (4 ft) in length. Ears develop above a few of the leaves in the midsection of the plant, between the stem and leaf sheath, elongating by ~ 3 mm/day, to a length of 18 cm (7 inches) to 60 cm (24 inches) being the maximum observed in the subspecies.

The female inflorescences, tightly enveloped by several layers of ear leaves are commonly called husks. The apex of the stem ends in the tassel, an inflorescence of male flowers. When the tassel is mature and conditions are suitably warm and dry, anthers on the tassel dehisce and release pollen. Maize pollen is anemophilous (dispersed by wind), and because of its large settling velocity, most pollen fall within a few meters of the tassel. Elongated stigmas, called silks, emerge from the whorl of husk leaves at the end of the ear. They are often pale yellow and

7 inches (178 mm) in length, like tufts of hair in appearance. At the end of each is a carpel, which may develop into a "kernel" if fertilized by a pollen grain.

The pericarp of the fruit is fused with the seed coat referred to as "caryopsis", typical of the grasses, and the entire kernel is often referred to as the "seed". The cob is close to a multiple fruit in structure, except that the individual fruits (the kernels) never fuse into a single mass. The grains are about the size of peas, and adhere in regular rows around a white, pithy substance, which forms the ear (maximum size of kernel in subspecies is reputedly 2.5 cm. An ear commonly holds 600 kernels. They are of various colors *viz.*, blackish, bluish-gray, purple, green, red, white and yellow. It lacks the protein gluten of wheat and, therefore, makes baked goods with poor rising capability (Kadam *et al.*, 2012). Young ears can be consumed raw, with the cob and silk, but as the plant matures the cob becomes tough and the silk dries to inedibility.

Yield is a complex polygenic character depending on number of characters influenced by the genotype-environment interaction. The genetic improvement primarily depends upon the nature and magnitude of variability in plants characters, overall genetic diversity among genotypes and association between characters. Therefore, it is necessary to analyze or examine the genetic diversity provided by the gene pools and then harnessed for further crop improvement.

The literature on genetic diversity is reviewed under following headings:

i. Genetic variability
ii. Correlation and path coefficient analysis, and
iii. Genetic divergence

2.1.1 Genetic Variability

Possibility of achieving improvement in any crop plants mainly depends on the magnitude of genetic variability. Phenotypic variability expressed by a genotype or a group of genotypes in any species can be partitioned into genotypic and phenotypic components. The genotypic component being the heritable part of the total variability, its magnitude for yield and its component characters influence the selection strategies to be adopted by the breeders. Researchers collect the germplasm from various agro-climatic regions and then evaluate and categorize them for genetic variability. The search for individual differences plays a significant role in improving

yield and quality of the crop through searching of new and diverse genetic resources. The psychology of individual differences can be studied by the concept of heritability.

Heritability is the proportion of phenotypic variance that is attributable to genetic variation among individuals. Heritability is the extent to which individual differences at genetic level contribute to individuals differences in observed behaviour (phenotypic individual differences). Both genetic and environmental factors contribute towards variation among individuals. Heritability plays a pivotal role in analyzing estimate of relative contributions of differences in genetic and non-genetic factors to the total phenotypic variance in a population. Phenotypic and genotypic variance, heritability and genetic advance have been used to assess the magnitude of variance in maize breeding material. Sharma *et al.* (1982), Saha and Mukherjee (1985), Tyagi *et al.* (1988), Singh *et al.* (1991), Rahman *et al.* (1995), Umakanth and Khan (2001), Srivas and Singh (2004), Abirami *et al.* (2007), Akbar *et al.* (2008) and Khodadad *et al*. (2013) also recorded variations in their material of maize.

Prakash *et al.* (2006) performed a study to identify QPM (quality protein maize) lines having high protein, oil and grain yield. Relative performance and genetic behavior for 14 yield and quality parameters were studied in 169 genotypes. The analysis of variance revealed significant variability for all the agronomic and quality parameters. Among the fourteen characters studied, the genetic advance as per cent of mean along with higher values of heritability, GCV and PVC estimates was maximum for grain yield per plot and protein yield, indicating that, the genetic variance for these two traits are probably due to their high additive gene effects.

Ahmad *et al.* (2011) conducted an experiment to estimate the genetic variability in 14 different maize genotypes. The results of analysis revealed that all the characters like days to 50% pollen shedding, days to 50% silking, plant height, ear height, 100 grain weight, harvest index and grain yield were significantly affected due to various maize genotypes.

Idris and Abuali (2011) evaluated nine open-pollinated maize genotypes to assess the magnitude of genetic variability for vegetative, yield and yield components under field conditions. They found non-significant differences for most character under study, except for plant height, stem diameter, rows per cob and ear length in the first season and for days until 50% flowering and 100-seed weight in the second season. Data recorded for heritability showed that stem diameter had maximum

heritability (67.02%) in the first season while the maximum heritability (84.57%) was recorded for days to 50% flowering in the second season. The study revealed considerable amount of diversity among the tested populations which could be manipulated for further improvement in maize breeding.

Atif and Mohammed (2012) observed significant variability for plant height, stem diameter, rows number and ear length in diverse maize lines during the first season 2007/08 and for days to 50% flowering and 100-seed weight during the second season 2008/09. Data recorded for heritability showed that days to 50% flowering had maximum heritability (79.1%) while the minimum heritability (4.46%) was recorded for 100-seed weight. The study revealed considerable amount of diversity among the tested populations which could be manipulated for further improvement in maize breeding.

2.1.2 Correlation and Path Coefficient Analysis

It is assumed that yield is a contribution of several characters which are correlated among themselves and to the yield. Correlation coefficient, which measures the extent of their relationship, indicates the strength and direction of a linear relationship between two random variables. It is a measure of the mutual relationship between various plant characters and determines the components on which selection can be based for improvement. The correlation refers to the departure of variables from independence, in broader sense there are several coefficients, measuring the degree of correlation, as specifically adapted to the nature of data. The association of characters may be due to either linkage or pleotropy. The knowledge of correlation that exists between important characters may facilitate proper interpretation of results and provide a basis of planning more efficient programmes.

The extent of observed relationship between two characters is known as phenotypic correlation. Genotypic correlation, on the other hand, is the inherent association between characters. The knowledge of interrelationship of various components on yield is used for different situations. The best known is the Pearson product-moment correlation coefficient, which is obtained by dividing the covariance of the two variables by the product of their standard deviation. The correlation concept was elaborated by Fisher and Yates (1963). The main results of the correlation is known as correlation coefficient and denoted by the symbol “r” which

ranges from -1.0 to 1.0. The closer r is to +1 or -1, the more closely the two variables are related. For no relationship between the variables, the value of r is close to 0.

Yield is a contribution of several characters which are correlated among themselves and to the yield, path coefficient analysis was developed (Wright, 1921). Unlike the correlation coefficient which measures the extent of relationship, path coefficient measures the magnitude of direct and indirect contribution of a component character to a complex character and it has been defined as a standardized regression coefficient which splits the correlation coefficient into direct and indirect effects. Path coefficient analysis provides better means for selection by resolving the correlation coefficient of yield and its components into direct and indirect effects.

Work done pertaining to correlation and path coefficient analysis in maize was earlier reported by Singh *et al.* (1999), Vaezi *et al.* (2000), Guang Cheng *et al.* (2002), Viola *et al.* (2003), Patel *et al.* (2005), Abirami *et al.* (2007), Akbar *et al.* (2008), and Mahesh *et al*. (2013).

Umakanth and Khan (2001) observed in diverse maize lines that grain yield per plot showed significant and positive correlations with ear circumference, ear length, plant height and 100-seed weight. Path analysis revealed that plant height followed by number of seeds per row, 100-seed weight, and ear length and ear circumference showed maximum positive direct genotypic effects as well as indirect contribution through other characters on grain yield.

Ei-Shouny *et al.* (2005) showed grain yield per plant in maize correlated positively and significantly with ear diameter, ear length, kernels per row, 100-kernel weight, rows per ear, ear height, plant height and days to silking under normal planting date and with kernels per row, ear diameter, 100-kernel weight, ear length, rows per ear, ear height and days to silking under late planting date.

Sumathi *et al*. (2005) observed the genotypic correlation of ear weight, rows per ear, kernels per row, and kernels per ear were positively associated with grain yield in maize genotypes. Oil per cent exhibited negatively non-significant correlation with grain yield, whereas it showed positive association with rows per ear only. Path coefficient analysis revealed that kernels per row showed high direct effect on grain yield followed by 100-seed weight, rows per ear and total kernels per plant.

Wali *et al.* (2006) revealed that grain yield was positively associated with plant height, ear length, ear circumference, kernels per row, fodder yield per plot and 100-grain weight, but was negatively correlated with number of days to 50% silking

at the phenotypic and genetic levels in *Z. mays*. The grain yield per plant was positively associated with plant height, ear length, ear circumference, kernels per row, fodder yield per plot and 100-grain weight at the phenotypic and genetic levels.

Heping *et al.* (2006) studied 42 maize inbreeds to assess the genetic variability parameters. Results showed that grain yield was significantly correlated with plant height, ear diameter, ear length, 100-kernel weight and grain production rate. Grain yield was most highly correlated with ear diameter, followed by 100-kernel weight, plant height, and ear length and grain production rate.

Abirami *et al.* (2007) indicated that grain yield showed positive association with oil and protein content. Path analysis showed that weight of the cob contributed a maximum direct effect to grain yield. It was implied that selection for weight of the cob would be highly effective for improvement of grain yield.

Sofi and Rather (2007) reported that the genotypic correlation coefficient revealed that ear diameter, 100-seed weight, ear length, kernel rows per ear and kernels per row showed the greatest correlation with grain yield. Path analysis indicated that 100-seed weight had greatest direct effect on grain yield, followed by kernels per row, kernel rows per ear, ear length and ear diameter.

Akbar *et al.* (2008) noticed in 32 maize genotypes that plant height had highly significant genotypic and phenotypic association with cob height and days to 50% tasseling with days to 50 per cent silking. All traits had significant genotypic association but not significant phenotypic association with grain yield.

Oktem (2008) showed a significant and positive correlation with single ear weight, ear length and ear diameter in maize. Ear length gave highest direct positive effect on fresh ear yield, followed by single ear weight. Plant height and stem diameter had negative direct effect on fresh ear yield. Direct effects of ear length, single ear weight, plant height and stem diameter on fresh ear yield were 42.3%, 31.3%, 31.0% and 17.7%, respectively.

Vaghela *et al.* (2009) studied the genotypic and phenotypic correlation of baby corn yield with different yield components, estimated from 54 diverse genotypes of maize (*Zea mays* L). The genotypic correlation coefficients were similar in direction of phenotypic correlation and higher in magnitude than phenotypic correlation coefficient.

2.1.3 Genetic Divergence

The assessment of genetic diversity using quantitative traits has been of prime importance in many contexts, particularly, in differentiating well defined populations. To help the breeder in the process to identify the parents that nick better, several methods of divergence analysis based on quantitative traits have been proposed to suit various objectives. These methods are measures of dispersion, components of genetic variances, metroglyph analysis and D^2 analysis. Among them, Mahalanobis's techniques based on the multivariate has been observed to be a good method of genetic stocks into various genetically diverse groups or clusters and making meaningful interpretation about genetic divergence in the germplasm. Genetic divergence analysis was carried out in maize lines by Castanon *et al*. (1999), Yin *et al.* (2004), Singh *et al*. (2005), Liu *et al.* (2006), More *et al*. (2006), Chen *et al*. (2007), Ganesan *et al.* (2010) and Khodadad *et al*. (2013).

Yin *et al.* (2004) studied cluster analysis for various plant traits (including plant height, ear height, tassel length, stem diameter, ear length, ear diameter, rows, grains per row, 100-grain weight, yield per plant, dried ear weight and maturity period) classified some 110 maize inbreds into 5 groups. The genetic diversity among the inbred groups was greater than that within the same group, and heterosis among the groups was greater than that within the same group. To breed outstanding crosses, the parents should be selected from the various groups.

Singh *et al*. (2005) estimated D^2 analysis using 23 genotypes of maize for various yield attributes including 50% tasselling, 50% silking, plant height, cob height, days to maturity, cob girth, cob length, rows per cob, grains per row, 100-grain weight and grain yield per plant. The genotypes fell into 6 clusters. The inter-cluster distances were higher than intra-cluster distances, suggesting wide genetic diversity among the genotypes of different groups. The inter-cluster D^2 values indicated the maximum distance between clusters III and VI and the lowest distance between clusters I and IV. The cluster means were higher for 50% tasselling, 50% silking, plant height, cob height, cob length, grains per row and 100-grain weight in cluster IV; for cob girth, days to maturity and rows per cob in cluster II; and for grain yield per plant in cluster III followed by cluster II.

Liu *et al*. (2006) studied 24 maize varieties for various quantitative traits and reported that cumulative contribution percentage of 7 principal components (morphology and yield factor, growth duration factor, rows/ear factor, low yield factor, oil content factor, protein content factor and ear length factor) to variation reached 83.14 per cent. Based on the analysis for each principal component vector, the introduced 24 varieties were grouped into 6 clusters.

More *et al.* (2006) studied 45 diverse genotypes of forage maize for genetic diversity and identify the suitable genotypes for hybridization programmes based on clustering pattern. The genotypes were grouped into 7 clusters using Mahalanobis D^2 statistics. Cluster II was the largest with 25 genotypes followed by cluster III with 11 genotypes and cluster I with 5 genotypes. The clusters IV, V, VI and VII were monogenotypic. The maximum inter-cluster distance was observed between clusters I and VI followed by distance between clusters I and IV and clusters I and V. Clusters V and VI exhibited the minimum inter-cluster distance.

Chen *et al*. (2007) used 186 maize hybrids for genetic divergence and classified into ten clusters, with 88.20 per cent of the hybrids included in Cluster 4, Cluster 8 and Cluster 10. The analysis of pedigree sources of 51 hybrids showed that 36 hybrids had close genetic relationships. It was indicated that the similarity was high and the genetic diversity was narrow among the 186 hybrids. It is necessary to broaden the genetic basis of breeding germplasm in maize.

Gautam (2008) reported the genetic divergence using D^2 statistic of 135 populations of maize (*Zea mays* L.) from different agro climatic situations and revealed existence of considerable diversity. The populations were grouped into 15 clusters. The cluster II was the largest containing 24 populations followed by cluster IV and I with 22 and 20 entries, respectively. The grouping of populations in cluster did not show any relationship between genetic divergence and geographic diversity.

Nehvi *et al.* (2008) observed high genetic divergence among 50 local maize cultivars through multivariate analysis following D^2 statistics. Cultivars were grouped into eight distinct clusters. Intra and inter cluster distances among the groups were determined and analyzed with respect to cluster means. Maximum inter-cluster distance was recorded between cluster 6 and cluster 7. Cluster mean analysis indicated that the clusters containing solitary cultivars mostly differed for all the traits barring ear length. Ear length followed by grain yield contributed maximum to the divergence. Tremendous potential exists for introgression of allelic resources present

in these adopted local cultivars into existing potential high yielding composites for recovery of high yield in recombinants.

Patel *et al*. (2009) conducted an experiment with 54 composites/hybrids baby corn genotypes. The data on baby corn yield and its attributes were subjected to Mahalanobis (1936) D^2 analysis using Tocher method for clustering. The results indicated that the varieties were distributed in seven clusters of which cluster V had maximum intra-cluster distance (14.89) and minimum in cluster VI (5.45). The composites/hybrids belonging to clusters V and VI were all from same geographical region. The inter-cluster distance ranged from 16.03 (clusters III and VI) to 60.05 (clusters IV and VII). Among clusters, variation was higher in green fodder yield, ear height, plant height and baby corn yield. Plant height and ear height were found to be the most important characters contributing to the total genetic divergence.

Ganesan *et al.* (2010) assessing the genetic diversity for 105 adapted maize germplasm lines were subjected to Mahalanobis D^2 analysis based on four characters *viz.*, plant height, cob height, cob length and kernel rows per cob in order to assess the genetic divergence among them. The analysis grouped the genotypes into four clusters. The percent contribution of each trait for total divergence revealed the highest contribution from plant height followed by ear length, kernel rows per cob and ear height. The maximum intra-cluster distance was observed in cluster II followed by cluster I and IV. The highest inter cluster distance was observed between cluster III and cluster IV followed by cluster I and III indicating greater variability in genetic make up of the genotypes included in these clusters. Based on inter cluster distances, genotypes present in the clusters *viz.*, I, III and IV are advisable to be used as parents for hybridization programme to develop heterotic hybrids in maize.

Yadav and Singh (2010) studied a set of maize inbred lines to compare how morphological and physiological characterization and RAPD molecular markers described variety relationships. All the inbred lines were confirmed as morphologically and physiologically distinct. At morphological level the maximum genetic distance (10.8) and least genetic distance (1.6) were found. For physiological characters, distance varied from 0.35 to 1.92 and results from dendrogram, which was made on the basis of dissimilarity matrix, were grouped into five major clusters. For RAPD, random primers provided polymorphic amplification products; the distance

varying from 0.42 to 0.65. The dendrogram showed that these lines formed close clusters due to less variation in these lines at molecular level.

Reddy *et al.* (2012) evaluated genetic divergence among 50 genotypes of maize as estimated by using Mahalanobis D^2 statistic for 11 characters. The genotypes were grouped into eight clusters. Cluster III was the largest with 20 genotypes followed by cluster I (12 genotypes), cluster II (8 genotypes), cluster IV (4 genotypes), cluster VIII (3 genotypes) and cluster V, VI and VII containing one genotype each. Based on the inter-cluster distances, genotypes present in clusters I, II, IV, V, VI and VII could be used as parents for hybridization programme to develop potential hybrids.

Rotili *et al.* (2012) used 81 maize genotypes to evaluate genetic diversity. The characters assessed were plant height, ear height, ear length, ear diameter, weight of 100 grains, hectoliter weight and grain yield. Genetic divergence was assessed by multivariate procedures, Mahalanobis distance, clustering methods of Tocher and nearest neighbor. The grains yield was major contributor to genetic diversity and ear length was the least.

Simon *et al.* (2012) estimated genetic divergence among 19 corn hybrids for characters like plant height, ear height, length of tassel, leaf area index, kernels rows per ear, kernels per row, thousand grain weight and grain yield. The genetic divergence was estimated from Mahalanobis dissimilarity and the hybrids were grouped by the methods of Tocher and non-weighted arithmetic averages (UPGMA). They showed greater genetic divergence between hybrids 2A525 and DKB177 and between P30K64 and DKB177. The indices of dissimilarity observed suggested that greater heterosis could be achieved by crossing lines from the hybrids AG8060, P30F35, 2B587, AG7088 and DKB390 with lines from the hybrids AG7000 and DKB177.

Udaykumar *et al.* (2013) evaluated 79 maize inbred lines and 3 checks and observations were recorded for 13 quantitative traits. Analysis of variance revealed highly significant difference among all inbred lines. Inbred lines were grouped into fourteen clusters, indicating the presence of genetic diversity based on D^2 analysis. The maximum inter cluster distance was observed between clusters II and XII (22.41) and highest intra cluster distance occurred in cluster XII (5.46) and also a wide range of variation was observed in cluster mean performance for the characters studied.

These genetically diverse inbred lines could be further used for developing superior hybrids and can also be utilized in developing synthetics and composites.

2.2 Biochemical Markers

The use of biochemical markers involves the analysis of seed storage proteins and isozymes. The technique utilizes enzymatic functions and is a comparatively inexpensive yet powerful method of measuring allele frequencies for specific genes. Allozymes, being allelic variants of enzymes, provide an estimate of gene and genotypic frequencies within and between populations. This information can be used to measure population subdivision, genetic diversity, gene flow, genetic structure of species and comparisons among species (out crossing rated), population structure and population divergence, such as in the case of crop wilds relatives. Major advantages of these types of markers consist in assessing co-dominance, absence of epistatic and pleotropic effects, ease of use and low costs.

Critical studies of selected enzymes reveal that each enzyme is polymorphic *i.e.,* consisting of related structures which catalyze the same general reactions but the members of each family, the isozymes differ in amino acid composition. Changes in the sequences and substitution of amino acids for one another in the enzyme are due to changes in gene sequence in DNA which affect the net changes and consequent mobility of the enzyme on a native electrophoretic gel. Activity staining of the seperated isozyme together with an appropriate dye will bring out differences in isozyme polymorphism which are in effect a measure of variation at the DNA level in the locus (or loci) coding for enzyme.

Isozymes have been the most commonly used biochemical markers in a large number of plant species to complement morphological characterization and to evaluate genetic variability among taxa. Each allozyme can be associated with a different allele of the same locus and, since its inheritance is co-dominant, differences in isozymes between two genotypes are related with their genetic similarity (Royo and Itoiz, 2004). The greatest drawback of these markers is their limited number and low level of polymorphism yet they are widely used in plant genetic research.

Wang and Chen (2005) used 23 isolates of maize to assess the biochemical diversity. They were clustered genotypes based on their soluble protein and isoenzyme profiles obtained through native polyacrylamide gel electrophoresis. A

significant diversity was found in terms of spectrum change of soluble protein and isenozyme including SOD (superoxide dismutase), MDH (malate dehydrogenase), PPO (phosphatidate phosphohydrolase), POD (peroxidase), EST (esterase) and CAT (catalase) among genotypes, and the obvious difference in the numbers of bands and activities of bands with the same Rf value were also detected. These data strongly supported that isoenzyme polymorphism could be indicators to reveal the pathogen diversity.

Markovic *et al.* (2010) studied two *Z. mays* L. inbred lines differing in drought tolerance subjected to osmotic stress induced by polyethylene glycol (PEG) treatment. Plant growth, as well as the changes in the isoenzymes of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD) in roots were analysed. Isoenzymes patterns obtained by isoelectric focusing of the studied enzymes demonstrated genotypic specificity. PEG treatment induced a decrease of all SOD and POD isoforms, except for one POD anionic isoform which could be involved in growth regulation.

Yan *et al.* (2010) analyzed isozymes pattern of peroxidase (POD), catalase (CAT), esterase (EST) and superoxide dismutase (SOD) on transgenic maize (with external chitinase gene) and its parent by vertical polyacrylamide gel electrophoresis. Results showed that, POD and EST were detected in 6 bands. POD-2 and POD-3 were present at the bud and seedling stages. POD-1, POD-4, POD-5 and POD-6 were only present at the seedling stage. POD-6 expressed stronger in the transgenic maize with chitinase than in its parent. EST-2 was present only at the bud stage, and its expression in transgenic maize was stronger than that in its parent. EST-5 only existed at the seedling stage. Four bands were detected for CAT; CAT-1 and CAT-3 were weaker bands than the others. CAT-3 in transgenic maize was stronger than in its parent. Three bands of SOD were detected; SOD-1 and SOD-2 existed at the bud and seedling stages, but SOD-3 was not shown in buds of the parent corn. All data showed that the expression of isozymes in transgenic and parent maize had obvious differences.

Wang *et al.* (2011) used esterase isozymes of different maize varieties analyzed by polyacrylamide gel electrophoresis. The results showed rich expression and strong specificity in enzyme spectrums of all the varieties, and there were stable and consistent bands of basic enzymes in many alleles. There were 76 enzyme bands in total for experiment materials. The numbers were significantly different between

varieties, 10 for the largest and 5 for the least. Some varieties possessed similar enzyme sites, but there were specific enzyme bands in various types due to different degree of staining.

2.3 Molecular Markers

Morphological and biochemical markers used for discriminating cultivars/varieties are not adequate under environmental influences, whereas the molecular markers have proven better. Molecular markers may or may not correlate with phenotypic expression of a genomic trait. They offer numerous advantages over conventional, phenotype-based alternatives as they are stable and detectable in all issues regardless of growth, differentiation, development or defense status of the cell. Additionally, they are not confounded by environmental, pleotropic and epistatic effects. They find a number of applications in crop improvement such as varietal identification, gene tagging, study of diversity among naturally occurring populations and those where diversity is most could be conserved. Regions that reveal maximum diversity or hot spot for the genotype can be brought under protective cultivation. Germplasm management could be brought about by conserving minimum number of plants which show maximum diversity. Diversity among individuals using molecular markers is revealed through a dendrogram which indicates how closely individuals are related. Diverse individuals with superior characters can be used in hybridization programmes so as to obtain maximum heterosis.

Molecular markers can help breeders to improve their breeding strategies and ease their efforts in producing superior varieties in shorter period of time. Even since their discovery (Botstein *et al.,* 1980), DNA markers have been extensively used in the improvement of crops, but their use in the improvement of medicinal plants is still being explored.

2.3.1 Randomly Amplified Polymorphic DNA (RAPD)

RAPD process involves amplification of DNA by using random arbitrary primers in a polymerase chain reaction. Primers are decamer (10 nucleotide length) single-stranded oligonucleotides of known sequence, which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way (Williams *et al.* 1990). It is most commonly used due to their efficient and quick assay for polymorphism analysis. They do not require prior sequence information and

require relatively small amounts of DNA. Assessment of molecular diversity using RAPD techniques in maize genotypes was also carried out by Hahn *et al.* (1995), Shuchen *et al.* (1995), Rafalski *et al.* (1997), Liu *et al.* (1998), Shieh and Thseng (2002), Garcia *et al.* (2004) and Mukharib *et al.* (2010).

Moeller and Schaal (1999) studied genetic variation among 15 accessions of Native American maize using random amplified polymorphic DNA. RAPD patterns revealed very high levels of polymorphism among accessions. Banding patterns ranged in percentage polymorphism from 46.7% to 86.2% with an overall mean of 70.7% for the primers analyzed. The construction of genetic relationships using cluster analysis and principal co-ordinates analysis revealed that RAPDs are successful in confirming hypothesized relationships and in identifying misclassified specimens.

Shieh and Thseng (2002) evaluated the genetic diversity of 13 maize inbred lines and determine the correlation between genetic distance and single cross hybrid performance, using RAPD markers. Forty different primers were used to give a total of 646 reproducible amplification products, 547 (84.7%) of them being polymorphic. Cluster analysis showed that the 13 inbred lines could be classified into distinct heterotic groups. There was no significant linear regression of grain dry weight heterosis value and mean performance of hybrids on genetic distance.

Carvalho *et al.* (2004) examined the genetic relationships among 81 maize accessions using RAPD markers. 32 highly informative primers amplified 255 markers of which 184 (72.2%) were polymorphics. Based on the RAPD markers, a dendrogram was constructed using the UPGMA method. The range of genetic similarity was from 0.78 to 0.91.

Valdemar *et al.* (2004) studied genetic relationships among 81 maize accessions consisting 79 landraces and two improved varieties in southern Brazil using RAPD. Thirty-two highly informative primers amplified 255 markers of which 184 (72.2%) were polymorphics. Based on the RAPD markers, a dendrogram was constructed using the UPGMA method. The range of genetic similarity was from 0.78 to 0.91. The molecular data grouped the accessions into two main clusters, which were correlated according to kernel colours.

Garcia *et al.* (2004) evaluated 18 inbred tropical maize lines using a number of different loci as markers. The loci used were 774 AFLPs, 262 RAPDs, 185 RFLPs and 68 SSR. For estimating genetic distance the AFLP and RFLP markers gave the

most correlated results, with a correlation coefficient of r = 0.87. Bootstrap analysis was used to evaluate the number of loci for the markers and the coefficients of variation (CV) revealed a skewed distribution.

Souza *et al*. (2008) used RAPD and SSR markers to compare genetic diversity among the 16 maize inbred lines. 22 primers were used in the RAPD reactions, resulting in amplification of 265 fragments. The similarity based on Dice coefficient for the RAPD ranged from 53 to 84%. The dendrogram obtained by from RAPD analysis showed five groups. The RAPD were effective to validate pedigree data.

Mukharib *et al*. (2010) identified the extent and distribution of genetic diversity by using randomly amplified polymorphic DNA marker in a selected group of maize inbred lines. A high level of polymorphism of 73.02 per cent was detected among the genotypes. The maximum genetic distance of 29.7 per cent was detected between CM-202 and KDMI-16. The minimum genetic distance of 12.8 per cent was observed between KDMI-04 and CI-05.

Cholastova *et al*. (2011) analyzed the molecular genetic diversity among 30 maize hybrids by RAPD and SSR markers. The RAPD system provided an average PIC of 0.71 (ranging from 0.47 to 0.91) and RAPD provided an average value of 0.61 (ranging from 0.44 to 0.82). Genetic similarities were estimated using Nei and Li's coefficients for SSR and Jaccard's coefficient for RAPD. For SSR, genetic similarity ranged from 26.3 to 88.5% (with average of 58.8%); for RAPD, GS ranged from 6.7 to 86.7% (with average of 49.5%). Hybrids were clustered using unweighted pair group method with arithmetic mean (UPGMA). The correlation between similarity matrices for RAPD and SSR was 0.11.

Nkongolo *et al.* (2011) assessed the level of genetic variation and relatedness among and within QPM and normal maize varieties using ISSR and RAPD markers. For ISSR analysis, the mean level of polymorphism within each accession analyzed was 69%. Nei's gene diversity (h) was 0.26 on the average. The genetic distance among the accessions varied from 0.098 to 0.460. More than 80% of genetic distances were below 0.35. The mean level of polymorphic loci among accessions obtained with RAPD markers was higher (79%) compared to ISSR. The genetic distance values were also larger than ISSR data ranging from 0.16 to 0.61 with 50% of values smaller than 0.35. The mean level of polymorphisms within the accessions analyzed was 65%. The Nei's gene diversity (h) was 0.23. Overall, the genetic variation among

and within QPM and normal varieties were high, but the genetic distance among them was small.

2.3.2 Inter Simple Sequence Repeats (ISSR)

ISSR are arbitrary multiloci markers produced by PCR amplification with a micro satellite primer discovered by Zietkiewicz *et al.* (1994). These are semi-arbitrary markers amplified by PCR in the presence of one primer complementary to target micro-satellite. Like RAPDs, ISSR markers are quick and easy to handle and no primer genomic information is required for their use and have been used for a number of plant species. ISSR markers overcome the short coming of the low reproducibility of RAPD; the high cost of AFLP, the complexity of SSR and represent a fast and a cost-efficient technique (Kurane *et al.* 2009). ISSR analysis in maize genotypes were also reported by Reddy *et al.* (2002), Chen *et al.* (2010) and Idris *et al.* (2012)

Domenyuk *et al*. (2002) assessed F_2 segregating maize populations using ISSR markers which were able to reveal a significant difference between alleles by a quantitative index. Confidence ranges have been determined for variation in 17 quantitative traits. Variations in the traits under study could be correlated with the inheritance of 16 marker loci.

Batovska *et al.* (2010) analyzed the polymorphism of maize lines using microsatellite and ISSR markers. Trinucleotide unanchored primer $(ATG)_6$ and dinucleotide primer $AGT(GT)_6$ anchored at 5' end primers have been used. Cluster analysis grouped lines had similar genetic background. The line T10 of unknown origin has been separated from the other tested lines at the value of genetic distance of 0.9 by using primer $(ATG)_6$. Meanwhile, by using the anchored primer the T10 line has been attached to the lines X4 (Iowa Stiff Stalk Synthetic) and B8 (lodent Reid x Lancaster Sure Crop x Iowa Stiff Stalk Synthetic) at the value of genetic distance 0.5. The polymorphism value ranged from 64% (unanchored primer) to 78% (anchored primer).

Chen *et al.* (2010) used three sweet maize hybrid combinations and their parents were involved to identify their seed purity by ISSR molecular marker technique. 40 primers were screened out from 30 ISSR primers to amplify genomic DNA of the 9 materials effectively and meanwhile 31 sites were amplified, including 24 polymorphism sites with a polymorphism rate of 77.4%. All of the 9 materials could be differentiated from each other based on the fingerprints established by these

specific bands. Systematical cluster analysis conducted with UPGMA showed that the 9 sweet maize materials could be divided into 2 groups.

Amaral *et al*. (2011) assessed 52 accessions of popcorn for variability analysis using ISSR markers. By using UPGMA clustering, a dendrogram was constructed and nine groups were made at a cutoff value of 0.36. Among the genotypes, the ancestors *Tripsacum sp*. and *Teosinte* were the most divergent, which corroborates the efficiency of the ISSR technique. *Teosinte* was in more proximity with maize than *Tripsacum sp*. Although, belonging to different heterotic groups, the dent and flint types of the common maize assembled in the group I confirmed that the compared analysis of genetic diversity was more remarkable.

Gupta *et al.* (2011) employed ISSR markers and protein profiling on SDS-PAGE to assess genetic diversity in four maize hybrids and their parents. Based on ISSR analysis, 3 primers were amplified and gave 35 scorable fragments with 100 percent polymorphism. ISSR profiling similarity matrix was obtained using Jaccard Similarity Coefficient that was observed up to 0.75. On this basis a dendrogram was constructed with UPGMA method. Twenty four scorable bands were resolved on 10% gel of which 22 bands were polymorphic (91.67%) and 19 scorable bands were resolved on 15% gel in which 17 bands were polymorphic (89.47%). In protein profiling, the value of Jaccard Similarity Coefficient lied between 0.21 to 0.65. According to the dendrogram of ISSR and protein profiling, hybrids resembled more with their female parent.

Vu-Van *et al*. (2011) characterized genetic diversity of local maize accessions using ISSR markers. Ten primers were used to study genetic diversity among 21 maize accessions consisting of 12 normal and 9 waxy maize accessions. A total of 108 ISSR fragments were detected and all of them were polymorphic (100%). PIC values of ISSR primers ranged from 0.10-0.39. The average PIC value for each primer was 0.24. The resolving power (Rp) value ranged from 0.48-14.29 with an average of 4.48 per primer. Based on UPGMA analysis, using 70% genetic similarity as the cutoff, a dendrogram was constructed and 21 maize accessions were grouped into three clusters. The similarity coefficients among accessions ranged from 0.52-0.90.

Idris *et al*. (2012) studied the extent and distribution of genetic diversity in crop plants using ISSR markers. A high level of polymorphism (of 69%) was detected among genotypes. The ISSR primers showed 10 fingerprints for six genotypes out of nine studied. The maximum genetic distance of 0.48% was detected between

Huediba-2 and Mogtama-45-2. While, the minimum genetic distance of 0.16 percent was observed between Giza-2 and Var.113. The results indicated that variation can be attributed to use of ISSR.

Mbuya *et al*. (2012) evaluate 137 inbred lines at molecular level using ISSR marker and revealed high level of genetic variability among inbreds. The level of polymorphic loci observed with ISSR markers varied between 74% and 80%. Nei's gene diversity and Shannon's information index values varied from 0.22 to 0.27 and from 0.34 to 0.41, respectively. The majority of inbred lines were distantly related. In fact, more than 87% of genetic distance values were above 0.50. The genetic distance values among the different parental maize accessions varied from 0.39 to 0.72. Inbred lines from the same parental accession were also not genetically close with genetic distance values varying from 0.28 to 0.59.

2.4 Sequence Characterized Amplified Region (SCAR) Marker

RAPDs are able to quickly and simply monitor marker loci but are not reliable because of their high sensitivity reaction conditions. The reproducibility of RAPD is affected by quality and concentration of template DNA, concentrations of PCR components and the PCR cycling conditions (Ellsworth *et al.* 1993). Subsequently, conversion of RAPD to SCAR markers can be a solution for the problem of irreproducibility (Paran and Michelmore, 1993) which can also be used as a physical landmark in the genome. These markers are co-dominant, mono locus and PCR-based that required two specific primers, having many advantages including their specificity, low cost, easy and fast use. This technique developed from RAPD fingerprints has been employed with success in plant and animal species identification (Mariniello *et al.* 2002; Yuan *et al.* 2007; Parasnis *et al.* 1999; Koveza *et al.* 2005; Arnedo-Andrés *et al.* 2002). In many cases, SCAR markers have been developed by converting RAPD markers. It involved cloning and sequencing of polymorphic RAPD fragment and then designing of SCAR primers based on the insert sequence containing the RAPD primer sequence. Conversion from RAPDs to SCARs lead to enhancement in reliability and efficiency, and therefore, has been used not only as physical landmarks in the genome but also as genetic markers (Paran and Michelmore, 1993).

Khampila *et al*. (2008) studied RAPD and SCAR markers associated with northern corn leaf blight (NCLB) resistance. Bulked segregant analysis (BSA) was

used to search for RAPD markers linked to NCLB resistance genes, using F_2 segregating population obtained by crossing a susceptible inbred '209W' line with a resistant inbred '241W' line. 222 decamer primers were screened to identify four RAPD markers: OPA07521, OPA16457, OPB09520, and OPE20536 linked to NCLB resistance phenotype. The RAPD and SCAR markers were developed successfully to identify NCLB resistant genotypes. Thus, the markers identified in this study should be applicable for marker assisted selection for the NCLB resistance in waxy corn breeding programmes.

Ladhalakshmi *et al.* (2009) developed a DNA sequence characterized amplified region (SCAR) marker for identification of isolates of *Pernospora. sorghi* from maize by using PCR. The RAPD primer OPB15 consistently amplified a 1,000 base pairs (bp) product in PCR only from DNA of *P. sorghi* isolates from maize and not from isolates of sorghum. The PCR-amplified 1,000-bp product was cloned and sequenced. The sequence of the SCAR marker was used for designing specific primers for identification of maize isolates of *P. sorghi*.

LuXiang *et al.* (2009) developed a SCAR marker using marker-assisted selection for disease-resistant breeding. Resistant and susceptible DNA bulks were composed by using DNAs of 10 resistant and 12 susceptible inbred lines, respectively. Polymorphic AFLP markers were screened between two bulks and then transformed into SCAR markers. These SCAR markers associated with sugarcane mosaic virus resistance was analyzed with disease incidence of 100 inbred lines. Two polymorphic AFLP markers, P66M38-220 and P55M51-240, were identified, and P66M38-220 was transformed into SCAR112, which was validated to be highly associated with SCMV resistance.

Shi *et al*. (2009) developed SCAR markers for map-based cloning of resistance genes and MAS. Two sets of BC3 progenies, one (BC3Q) derived from the cross Qi319 (resistance) x Huangzao 4 (susceptible), the other (BC3M) from Mo17 (resistance) x Huangzao 4 (susceptible), were generated. Huangzao 4 was the recurrent parent in both progenies. A combination of bulked segregant analysis with AFLP (amplified fragment length polymorphism) method was applied to map the genes involving the resistance to *S. reiliana*, and corresponding resistant and susceptible bulks and their parental lines were used for screening polymorphic AFLP primer pairs. One fragment of P13M61-152 was converted into SCAR (sequence charactered amplified fragment) marker S130.

Couillerot *et al.* (2010) assessed the applicability of SCAR markers obtained from BOX, ERIC and RAPD fragments to design primers for real-time PCR quantification of the phyto-stimulatory maize inoculants *Azospirillum brasilense* UAP-154 and CFN-535 in the rhizosphere. They designed primers based on strain-specific SCAR markers and were screened for successful amplification of target strain and absence of cross-reaction with other *Azospirillum* strains. The specificity of primers thus selected was verified under real-time PCR conditions using genomic DNA from strain collection and DNA from rhizosphere samples.

Zhang *et al*. (2010) employed a study to develop DNA markers closely linked to the resistance gene mdm1. Linkage between the markers and phenotypes was confirmed by analyzing an F_2 population obtained from a cross between a resistant parent 'Huangzaosi' and a susceptible parent 'Mo17 (478)'. Four AFLP markers were found in the maize dwarf mosaic resistant plants. By using bulked seggregant analysis, two of the four AFLP markers were transformed into SCAR, nominated Rsun-1 and Rsun-2. The two AFLP markers, RHC-1 and RHC-2, from the amplification products of primer combination E-AGC/M-CAA and E-AGC/M-GAA, showed linkage with the mdm1 gene at a genetic distance 1.6 and 2.0 cM, respectively.

Shi *et al*. (2011) conducted a study for marker development and marker-assisted selection as an efficient way for maize rough dwarf disease-resistant breeding. A total of 152 maize inbred lines, resistant and susceptible DNA bulks were composed by using genomic DNAs of 10 resistant and 10 susceptible inbred lines, respectively. Polymorphic AFLP markers were screened between two bulks and then transformed into SCAR markers. Identification of correlation between the SCAR markers and MRDD resistance was carried out in 152 inbred lines. They developed SCAR69 and SCAR74 that were validated to be highly associated with MRDD resistance.

3. MATERIALS AND METHODS

The present investigation entitled "Assessment of Morphological, Biochemical and Molecular Diversity in *Zea*. *mays* L." was carried out at the Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur (Rajasthan). Both field and laboratory experiments were conducted in order to achieve the objectives of present investigation.

3.1 Experimental Site

The field studies were undertaken at Instructional Farm and Laboratory studies were taken at Rajasthan College of Agriculture, Udiapur. Udaipur situated in South Eastern part of Rajasthan state, is located at an elevation of 579.50 meter above mean sea level on latitude of $24^0$35' North and longitude of $70^0$42' East. The climate of the region is sub-humid type with an average rainfall of about 637 mm and soil is sandy-loam. Crop was raised during *kharif*, 2011 and meteorological data recorded during period are presented in Table 3.1.

3.2 Experimental Material

In the present investigation seed of twenty diverse genotypes/varieties of *Z. mays L.* were procured from different geographical sources. The nucleus seed of five genotypes of high quality protein maize were obtained from CCHAU Hisar. Five hybrids and seven composites were procured, from Department of Plant Breeding and Genetics and remaining three local land races were obtained from NAIP, Biodiversity, PBG, Rajasthan College of Agriculture. The source and pedigree of material used are given in Table 3.2.

3.3 Field Studies

3.3.1 Experimental Design

The field experiment was laid out in randomized block design with three replications. Twenty genotypes were planted each in three rows of 15.6 m length with row to row distance of 70 cm. Fertilizers were applied @ 120 kg N: 70 kg P_2O_5 and 70 kg K_2O/ha at the time of sowing while 40 kg N/ha was top-dressed in three split

doses in twenty, thirty and fourty-five days respectively. Immediately after sowing a light irrigation was given. The crop was raised under normal rainfall condition with recommended agronomical practices and plant protection measures.

Table 3.1: Weekly meteorological data during the crop growth period (2011) at Udaipur

Week	Week No.	Temperature (^{0}C)		Relative Humidity (%)		Wind Velocity (kmph)	Sunshine (hrs)	Rain-fall (mm)	Evapo ration (mm)
		Max.	Min.	Max.	Min.				
08 July-15July	1	32.38	24.1	94.67	71.0	3.8	5.5	13.7	5.2
16 July-22 July	2	30.5	23.8	92.5	69.5	7.5	3.2	5.2	4.4
23 July-30 July	3	29.9	23.6	96.6	78.0	4.6	3.5	11.2	3.9
01 Aug-07 Aug	4	31.0	24.1	90.8	703	3.6	5.4	6.4	4.4
08 Aug-15 Aug	5	28.1	23.6	95.1	74.6	3.7	4.3	9.4	2.2
16 Aug-22 Aug	6	28.7	23.8	97.4	77.77	3.4	3.6	11.8	2.7
23 Aug-30 Aug	7	30.2	22.9	97.7	78.4	3.7	4.6	16.4	2.5
01 Sept- 07 Sept	8	29.4	22.6	98.5	78.5	3.8	2.9	12.2	2.1
08 Sept- 15 Sept	9	28.4	22.1	96.3	75.6	4.7	4.2	18.6	2.4
16 Sept- 22 Sept	10	29.3	21.7	93.9	73.2	3.9	5.1	3.6	2.3
23 Sept- 30 Sept	11	29.6	19.0	88.4	60.4	3.4	8.8	0.0	3.2
01 Oct – 07 Oct	12	31.1	18.6	79.6	55.5	3.4	9.2	0.0	4.3
08 Oct – 15 Oct	13	33.2	19.1	75.8	46.0	2.3	8.6	0.2	4.5
16 Oct – 22 Oct	14	32.9	17.4	73.9	34.98	1.8	8.1	0.0	4.0
23 Oct – 30 Oct	15	32.5	15.2	72.22	33.5	1.9	8.4	0.0	3.8
01 Nov–07 Nov	16	30.5	16.3	73.43	28.6	1.8	7.8	0.0	3.1

Table 3.2: Pedigree and source of 20 genotypes of *Z. mays* L. used for study

Genotype	Pedigree	Source
1. QPM		
HQPM-1	HKI-193-1 X HKI-163	CCS HAU, Hisar
HQPM-5	HKI-163 X HKI-161	CCS HAU, Hisar
HQPM-7	HKI-193-1 X HKI-161	CCS HAU, Hisar
EQH-16	Unknown	CCS HAU, Hisar
EQH-63	Unknown	CCS HAU, Hisar
2. Hybrids		
PHM-1	EI-116 X EI-634	MPUAT, Udaipur
PHEM-2	CM-137 X CM-138	IARI, New Delhi
PHM-2	EI-472 X EI-460	RCA (MPUAT) Udaipur
BIO-9637	Unknown	Bioseed Company
HM-8	HKI-163 X HKI-163	CCS HAU, Hisar
3. Composites		
Arawali	Bulk of early and stress tolerant HS families from X-2 W pool	RCA (MPUAT) Udaipur
PM-3	Bulk of CEW- 8 pool	RCA (MPUAT) Udaipur
PM-4	Bulk of material pool-2	RCA (MPUAT) Udaipur
PM-5	Bulk of II HS progenies selected from C3 cycle of material pool-2	RCA (MPUAT) Udaipur
Navjot	Pratap x Tarun	PAU, Ludhiana
PM-6	Compositing of 11 early to medium white seeded entries	RCA (MPUAT) Udaipur
EC-3161	Unknown	RCA (MPUAT) Udaipur
4. Local land races		
Black Sathi	Local land race	RCA (MPUAT) Udaipur

Kumbhalgarh Malan	Local land race	RCA (MPUAT) Udaipur
Chanawada Sathi	Local land race	RCA (MPUAT) Udaipur

3.3.2 Morphological/Agronomical Characters

Five competitive plants were randomly selected from each replication of a genotype for recording observations. The selected plants were tagged and data on individual plant were recorded for the following characters except for days to 50 per cent flowering and days to maturity. The procedure adopted for recording observations on different characters are given below:

1) **Plant height (cm):** The plant height was measured in centimeters from the base of the plant to the top of the main spike when crop is fully mature.
2) **Days to 50% flowering:** Number of days were counted from the date of sowing to the date on which 50% plants of a plot completed the opening tassel for each genotype.
3) **Cob length (cm):** Cob lengths of twenty plants were taken randomly when crop was 75-80 DAS, length was measured in centimeter for each cob and average was worked out.
4) **Cob girth (cm):** Cob girth of all randomly selected cobs was measured.
5) **Number of kernel rows per cob:** Number of kernel row borne by a cob and attained physical maturity were counted on randomly selected plants.
6) **Number of kernels per row:** Total number of kernels per rows were counted on randomly selected plants.
7) **Hundred grain weight (g):** Randomly 100 seeds were counted for each genotype and dried completely from each plot. The moisture content was 8 percent. The seed was weighed in grams using electronic single pan balance.
8) **Biological yield per plant (g):** The randomly selected plants from each plot were harvested separately, sun dried, weighed and average value was worked out to obtain biological yield per plant.
9) **Grain yield per plant (g):** Total grain obtained from selected plants were weighed in grams on electronic single pan balance and average was worked out.

10) **Harvest index (%):** Harvest index is the ratio of seed yield to biological yield in percentage and was calculated as per Donald (1962).

$$\text{Harvest Index} = \frac{\text{Seed yield per plant (g)}}{\text{Biological yield per plant (g)}} \text{X } 100$$

11) **Days to maturity:** Number of days were counted from the date of sowing to the date when 100% of the plants in a plot attained physiological maturity or their cob completely turned brown and leaves started senescence.

3.4 Laboratory Studies

3.4.1 Reagents and Chemicals

All reagents and chemicals used in the present investigation for enzyme extraction, staining of isozymes, DNA isolation, PCR amplification and electrophoresis were of high purity analytical grade and were purchased from SRL (Sisco Research Lab.), E. Merck, Himedia Laboratory and Banglore Genei Pvt. Ltd.

3.4.2 Biochemical Analysis

The changes in the protein profiles for isozyme activity on the basis of banding pattern for Esterase, Peroxidase and Superoxide dismutase was recorded from young leaves of 28 DAS.

3.4.2.1 Enzyme Extraction

Preliminary experiments were conducted to optimize the extraction condition with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and others constituents of extraction medium according to Sharma *et al.* (2008) with minor modification. Finally, the standardized extraction medium for esterase (EST), peroxidase (POX) and superoxide dismutase (SOD) consisted of 0.1 M Tris-Cl buffer (pH 7.5) containing 3% (W/V) polyvinylpyrrolidine (PVP), 1mM EDTA and 1mM $CaCl_2$. The crude enzyme was extracted from young leaves (28 DAS) by macerating 5 g tissue with 15 ml ice cold extraction medium in a pre-chilled pestle and mortar using acid washed sand as abrasive. The homogenate was filtered through four layered cheese cloth and filtrate centrifuge at 10,000 rpm for 10 min in a refrigerated centrifuge (Sigma 3K30) at 4^0C. The supernatant was transferred to microcentrifuge tubes and stored at -20^0C, until used for electrophoresis.

3.4.2.2 Total Soluble Proteins

Total soluble protein was estimated by Lowery's methods (Lowry *et al.*, 1951). To 0.1 ml of aliquot from respective crude extracts, 3.0 ml of alkaline copper sulphate solution (Appendix-I) was added with shaking and allowed to react for 10 minutes. To each tube, 0.3 ml of Folin Ciocalteau reagent (Appendix-I) was added, shaken, and incubated at room temperature for 30 minutes in order to complete the reaction. Absorbance of the blue colour was measured at 620 nm against a reagent blank. A standard curve was prepared using bovine serum albumin (1.0 mg/ml) as standard protein.

3.4.2.3 Polyacrylamide Gel Electrophoresis (PAGE)

Native-PAGE was performed using anionic system of Davis (1964).

Reagents

(A) Acrylamide-bisacrylamide solution

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Volume	100.0 ml

The solution was filtered through Whatman No. 1 filter paper and stored at 4^0C in brown bottle. The solution was stable upto 3 months.

(B) TEMED

Used as supplied, stored at cool and dry place, protected from sunlight.

(C) Ammonium persulphate solution (1.5%)

Ammonium persulphate	0.075 g
Volume (by dd water)	5.0 ml

The solution was prepared fresh before use.

(D) Stacking gel buffer (0.5 M)

Tris (amino-methane)	6.0 g
pH	6.8
Volume	100 ml

Added Tris in 50 ml of dd water, adjusted pH to 6.8 by concentrated HCl and final volume made to 100 ml.

(E) Resolving gel buffer (3 M)

Tris (amino-methane)	36.3 g

pH 8.8

Volume (by dd water) 100 ml

Added Tris in 50 ml of water, adjusted pH to 6.8 by concentrated HCl slowely and mixed the contents till pH became 8.8 and final volume made to 100 ml.

(F) Reservoir buffer

Tris 3.0 g

Glycine 14.4 g

pH 8.3

Volume (by dd water) 1L

Gel composition for native PAGE

Stock solution	**Stacking gel (ml) (3.75%)**	**Resolving gel (ml) (10%)**
Acrylamide-bis-acryalamide	1.25	10.0
Stacking gel buffer	2.50	-
Resolving gel buffer	-	3.75
1.5% APS	0.75	1.50
TEMED	0.025	0.060
Distilled water	5.47	14.70
Total	10.00	30.00

Procedure

Electrophoresis was carried out on slab gels using vertical midi slab gel electrophoretic system (Banglore Genei, India). Glass plates were washed with chromic acid, rinsed with distilled water and oven dried. The plates were wiped with an ethanol swab, air-dried and assembled in the gel casting assembly. Sides of the plates were sealed by Teflon spacers, clamped to make a mould and desired resolving gel solution was poured. A layer of water was then gently overlaid using a syringe. Polymerization of the gel took about 30 min and was indicated by a sharp interface between water and gel. Water was removed, stacking gel solution poured and comb inserted immediately with care so that no air bubble trapped beneath it. After polymerization, comb and lower spacer were removed. Gel plates were fixed to the electrophoresis apparatus, samples

containing about 45 µg proteins were loaded in separate wells and electrophoresis was carried out at a constant current of 50 mA for first 30 min followed by 70 mA constant current till the tracking dye reached one cm away from lower end of the gel.

3.4.3.4 Sample Preparation

To 400 µl sample (1µg/µl) of each genotype extract, added 15 µl of 5% bromophenol blue was added followed by 85 µl of 40% glycerol. The contents were mixed thoroughly and used for native PAGE.

3.4.3.5 Staining of Isozymes

Immediately after running electrophoretic unit, the side spacers were removed and mixed the glass plates were separated with the help of spatula. Gels were stained with specific staining solution as described below for each enzyme.

(A) Esterase

Gel: 10% resolving gel.

Staining: Esterase activity was localized on the gel according to Shaw and Prasad (1970) with minor modifications. 40 mg of α-naphthyl acetate was dissolved in 1 ml of acetone and the volume was made up to 100 ml with 100 mM phosphate buffer (pH 6.0). Then 76 mg of Fast Blue RR salt was dissolved by vigorous stirring. Gel was incubated at 35^0C for 20 minutes and washed with distilled water. Brown bands of esterase appeared almost immediately on a clear background.

(B) Peroxidase

Gel: 10% resolving gel.

Staining: Peroxidase activity was localized on the gel according to Guikema and Shermen (1980). The gels were stained in solution of 25% acetic acid containing 0.3% benzidine and 0.5% H_2O_2. Within 2 min, blue coloured bands appeared which turned brown after 10-15 min.

(C) Superoxide dismutase

Gel: 10% resolving gel.

Staining: Superoxide dismutase activity was localized on the gel according to Geburek and Wang (1990) with minor modifications. The gel was immersed in 40 ml of 0.2 M Tris-HCl (pH 8.0), 5 mM EDTA buffer containing 0.2 ml of 0.5 M $MgCl_2$, 1.0 ml of 1% aqueous nitro blue tetrazolium (NBT) and 0.5 ml of 1% aqueous phenazonium

methosulphate. The gel was exposed to strong fluorescent light for 15 minutes followed by one hour incubation in dark. Superoxide dismutase appeared as light bands (negatively stained) on a dark blue black ground. The gel was maintained in distilled water till photographed.

3.4.3.6 Scoring of Gels

Gels were visually scored by putting the gels on a light box. Bands with dark to very light intensities were scored and used to construct the zymograms. Rf (Rm=Relative mobility) value of each band was calculated using the following formula (Eeswara and Peiris, 2001).

$$Rm = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by the tracking dye}}$$

Bands were numbered on the basis of increasing Rf value or according to the distance travelled in the gel.

3.4.4 Molecular Analysis

3.4.4.1 Genomic DNA Isolation

Genomic DNA was extracted from young leaves of 21-28 DAS of *Z. mays* L. following CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method as described by Doyle and Doyle (1987)

Reagents

1. Leaf samples

   2 g of fresh leaf samples

2. Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction Buffer (100 ml):

CTAB	-	2% w/v
Tris HCl pH 8.0	-	100 mM
Sodium chloride	-	1.4 M
EDTA	-	20 mM

   (Autoclaved Tris, NaCl and EDTA. CTAB was added after autoclaving and extraction buffer was preheated before using).

3. Tris EDTA (TE) buffer:

Tris- HCl (pH 8.0)	-	10 mM
EDTA (pH 8.0)	-	1 mM

Dissolved and volume made up to 100 ml with dd water, autoclaved and stored at 4°C.

4. Ice cold Isopropanol
5. Chloroform: Isoamyl alcohol (24: 1 v/v)
6. Sodium acetate (3.0 M) pH 5.2.
7. Ethanol (70% and 100%)
8. RNAase A -10 mg/ml; Dissolved RNase A in TE and boiled it for 15 minutes at 100°C to destroy DNAase and store at –20°C

Protocol

1. Transfer 1 g of leaf segments into prechilled mortar, frozen using liquid nitrogen for 30 min. and grind it to fine powder.
2. The fine powder was allowed to thaw in the presence of 10 ml of pre-heated extraction buffer and incubated for 30-45 minutes at 65^0C with occasional mixing.
3. Add equal volume of Chloroform: Isoamylalcohol mixture (24:1 v/v) and mixed by inversion for 1 hour.
4. Centrifuged at 10,000 rpm for 20 minutes at room temperature.
5. Transferred the clear aqueous phase to a new sterile tube. Added equal volume of ice cold Isopropanol and mixed gently by inversion and then keep it in the freezer until DNA is precipitated out.
6. Using blunt end tips, the precipitated DNA into an eppendorf tube.
7. Air dryed the spooled DNA after removing the supernatant by brief spin.
8. Added 500 µl of TE to dissolve the DNA and then 10 µl of RNase and incubate at 37^0C for 30 minutes.
9. Add 500 µl of Chloroform: Isoamylalcohol mixture and centrifuged for 10 minutes.
10. Transfered aqueous phase to another eppendorf tube without disturbing the inner phase.
11. Added 2.5 volume of absolute Alcohol and 1/10th volume of Sodium acetate and kept for overnight incubation.
12. Centrifuged and discard the supernatant.

13. Used 500 µl of 70% and 100% ethanol subsequently to wash the DNA by centrifugation.
14. Discarded the Alcohol and removed the water from the DNA completely by air drying.
15. Dissolved the DNA pellet in 150-250 µl of TE (depending on the pellet size) and stored at 4^0C.

Purification of DNA:

Major contaminants in crude DNA preparation are RNA, protein and polysaccharides. Inclusion of CTAB in DNA extraction buffer helps in elimination of polysaccharides from DNA preparations to a large extent. The RNA was removed by treating the sample with DNase free RNase. Protein including RNase was removed by treating with the phenol: chloroform (1:1) and chloroform: isoamyl alcohol (24:1) subsequently. The purification was carried out in the following steps-

- RNase (50µg/ml) was added to crude DNA preparation and incubated at 37^0C for 1 hr.
- Equal volumes of phenol: chloroform in the ratio of 1:1(v/v) was added and mixed.
- Centrifuged the tubes for 5 min at 10000 rpm and supernatant was collected in another tube avoiding the whitish layer of inter face.
- Equal volumes of chloroform: isoamyl alcohol in the ratio of 24:1(v/v) mixed thoroughly for 15 minutes to form an emulsion.
- Centrifuged the tubes for 15 min at 15000 rpm and supernatant was collected in another tube avoiding the whitish layer of inter face.
- 0.1 ml of 3 M sodium acetate (pH=4.8) was added and mixed properly.
- The DNA was precipitated by addition of 2.5 times volume of absolute alcohol.
- The solution was centrifuged at 10000 rpm for 15 minutes. When pellet settled down, it was washed with 70 per cent alcohol and dried overnight.
- The DNA was dissolved in 200 µl of $T_{10}E_1$ buffer.
- The dissolved DNA was stored at -20 ºC.

3.4.4.2 Quantification of DNA

The optical density (Absorbance) of DNA was recorded at 260 and 280 nm by using a UV spectrophotometer in following steps-

- 2-5 µl of DNA diluted to 1000 µl of $T_{10}E_1$ medium in a cuvette was mixed properly and the optical density (OD) recorded at both 260 and 280 nm.
- The concentration of DNA was calculated by following formula –

$$\text{DNA Concentration (µg/µl)} = \frac{\text{OD (260) X 50}}{1000} \text{ X dilution factor}$$

- The quality of DNA was judged from the ratio of OD value recorded at 260 and 280 nm.

3.4.4.3 Agarose Gel Electrophoresis

Genomic DNA sample were resolved by submerged horizontal electrophoresis in 0.8 per cent (w/v) agarose gel and visualized by staining with ethidium bromide**.**

Reagents

(A) 10X TBE buffer

Tris	108.0g
Boric acid	55.0g
0.5 M EDTA (pH 8.0)	1L

(B) 6X Loading dye

Sucrose	4.0g
Bromphenol blue	0.025g
Xylene cynol	0.025g
Final volume	10 ml

Loading dye solution was stored at 4^0C.

Procedure

Gel casting tray was washed, air dried and its ends were sealed with tape. Agarose was melted by boiling in 1X TBE buffer, cooled to 60-65^{0}C and ethidium bromide (10mg/ml) at a concentration of 3µl/100ml was added. Gel solution was poured into gel casting plate with an appropriate comb with required number of wells and sizes inserted. Plate was submerged in 1X TBE buffer and comb was removed gently. Samples were prepared by adding 2µl of 6X loading dye and were spun briefly in a microcentrifuge for proper mixing. DNA samples (5 µl of samples and 1 µl 6X loading dye) were loaded in the wells and electrophoresis was carried out at constant voltage (3V/cm of gel) till

bromophenol blue/loading dye migrated to other end of the gel. The gel was visualized on a UV-transilluminator and photographed in a documentation system.

3.4.4.4 List of Primers

A total of twenty decanucleotide (10 nucleotide per primer) RAPD primers were used for PCR amplification. The sequences of these primers were selected from literature and synthesized from Bangalore Genei Pvt. Ltd., Banglore. The details of primer code sequence of the primer and GC contents are given in Table 3.3.

Table 3.3: Details of RAPD primers used in molecular analysis of *Z. mays* L. genotypes

S.No.	Primer*	Sequence 5' to 3'	G:C Content (%)
1	OPA-01	CAGGCCCTTC	70
2	OPC-08	TGGACCGGTG	70
3	OPD-05	TGAGCGGACA	60
4	OPD-12	CACCGTATCC	60
5	OPE-03	CCAGATGCAC	60
6	OPF-17	AACCCGGGAA	60
7	OPF-19	CCTCTAGACC	60
8	OPJ-04	CCGAACACGG	70
9	OPP-01	GTAGCACTCC	60
10	OPP-02	TCGGCACGCA	70
11	OPP-03	CTGATACGCC	60
12	OPP-04	GTGTCTCAGG	60
13	OPP-05	CCCCGGTAAC	70
14	OPP-06	GTGGGCTGAC	70
15	OPP-07	GTCCATGCCA	60
16	OPP-08	ACATCGCCCA	60
17	OPP-09	GTGGTCCGCA	70
18	OPP-10	TCCCGCCTAC	70
19	OPP-12	AAGGGCGAGT	60
20	OPP-16	CCAAGCTGCC	70

* Operon series code

Fifteen ISSR primers were also used for PCR amplification. The sequence of these primers were selected from literature and synthesized from Sigma Chemicals Co. U. S.A. The details of primer code sequence of the primer, G:C contents and melting temperature (Tm) are given in Table 3.4.

Table 3.4: Details of ISSR primers used in molecular analysis of *Z. mays* L. genotypes

S.	Primers	Sequence 5' to 3'	G:C	Tm (°C)

No.			Content (%)	
1	810	ATATATATATATATATT	0	24
2	851	GTGTGTGTGTGTGTGTYG*	50	54
3	802	ATATATATATATATATATG	6	25
4	852	TCTCTCTCTCTCTCTCCRA*	44	45
5	803	ATATATATATATATATC	6	24
6	853	TCTCTCTCTCTCTCTCRT*	44	47
7	804	TATATATATATATATAA	0	23
8	854	TCTCTCTCTCTCTCTCRG*	50	48
9	805	TATATATATATATATAC	6	22
10	855	ACACACACACACACACYT*	44	52
11	806	TATATATATATATATAG	6	22
12	856	ACACACACACACACACYA*	44	50
13	807	AGAGAGAGAGAGAGAGT	47	42
14	857	ACACACACACACACACYG*	50	54
15	808	AGAGAGAGAGAGAGAGC	53	47

* Degenerate primers, R = AT, Y = GA

3.4.4.5 Polymerase Chain Reaction (PCR) Amplification

PCR amplification was carried out in programmable thermal cycler from Eppendorf AG, Germany. PCR reaction for RAPD and ISSR markers was carried out in 20 µl of reaction mix containing 25 ng genomic DNA, 2 µl of 10X Taq DNA polymerase buffer (containing 1.5 mM $MgCl_2$), 200 µM each dNTPs, 0.03 µM of primer and 1 unit of Taq DNA polymerase in 200 µl Eppendorf tube.

The following protocols were used for PCR amplification:

For RAPD Primers:

PCR condition for RAPD analysis included an initial predenaturation step of 5 minutes at 94^0C and following 35 cycles of amplification.

Denaturation	94^0C	1minute
Annealing	36^0C	1minute
Extension	72^0C	2minute

→ 35 cycle

Final extension was carried out at 72^0C for 5 minutes and a hold temperature of 4^0C at the end.

For ISSR Primers:

PCR condition for ISSR analysis included an initial predenaturation step of 5 minutes at 94^0C and following 35 cycles of amplification.

Denaturation	94^0C	1minute
Annealing*	22.0^0-53.0^0C	1minute

→ 35 cycles

Extension 72^0C 2minute

*Annealing temperature varyied from 22.0^0-53.0^0C for different primers (Table 3.4).

Final extension was carried out at 72^0C for 5 minutes and a hold temperature of 4^0C at the end.

3.4.4.6 Allele Scoring

Following the PCR amplification, the PCR products of RAPD were loaded on 1.5% agarose gel while ISSR PCR products on 0.8 percent agarose gel, both of which were prepared in 1X TBE buffer containing ethidium bromide (10mg/ml) concentration of 3µl/100ml. Amplified PCR products were mixed with 2 µl of 6X gel loading dye and loaded in the wells of agarose. Electrophoresis was carried out at a constant voltage (3V/cm of gel) till bromophenol blue/loading dye migrated to other end of the gel. The gel was visualized on a UV-transilluminator and photographed using gel documentation system.

RAPD and ISSR amplification profile were scored visually, based on the presence (Taken as 1) or absence (Taken as 0) of bands for each *Z. mays* L. genotype. Only clear and unambiguous bands were scored. The size (in nucleotide base pairs) of the amplified bands was observed based on its migration and confirmation relative to standard molecular size markers (100bp DNA ladder and 1kb DNA ladder from Banglore Genei Pvt. Ltd., Banglore).

3.4.5 Development of Specific Sequence Characterized Amplified Regions (SCARs) Marker

3.4.5.1 Screening of RAPD Primers

Out of 20, sixteen random decamer primers (Operon Technologies, Inc.) produced polymorphic banding pattern were screened for diversity analysis. The polymorphic (distinct) band was identified among the selected maize genotypes. The primer OPP-05 showed distinct polymorphic band in all the quality maize protein varieties and absent in other genotypes. Thus this, amplicon was used for the development of SCAR marker for quality protein loci. PCR was repeated twice to ensure reproducibility and consistency of the banding patterns by OPP-5 primer.

3.4.5.2 Separation of Amplified Products by Agarose Gel Electrophoresis

About 100 µl of amplified products from each tube along with 10 µl of loading dye were separated on 1.2 percent agarose gel using 1x TAE buffer prepared from 50x TAE buffer at pH 8 along with 100 bp and double digest ladder (Bangalore Genie Pvt. Ltd, Bangalore) as DNA molecular weight marker. Electrophoresis was done at 50V for initial 30 min and then 70V for 1 hour and gel image was documented using gel documentation system.

3.4.5.3 Cloning of the RAPD Amplicon

The desired unique bands amplified in *Z. mays* L. was excised from sterile gel slicer and purified by Clean Genei Gel Extraction kit. Two microliters purified DNA was ligated into a to pTZ57R/T vector (2886 bp) according to supplier's instructions. The ligated vector was introduced into competent *Escherichia coli* strain DH5α following protocol for transformation by calcium chloride (Sambrook and Russell 2001). The distinct white colonies were picked up from the LB-ampicillin plate and recombinant DNA isolated from each overnight grown colony. Confirmation of the clones was done by restriction digestion of purified plasmid DNA.

3.4.5.4 Sequencing, SCAR Primer Designing and Amplification of the Genomic Region

The recombinant plasmid was purified by protocol of Sambrook and Russell (2001). Both ends of each DNA insert was sequenced. Based on the sequenced RAPD amplicon a pair of SCAR oligonucleotide primer pair was designed. The primer sequences were synthesized and used further.

3.5 Statistical Analysis

3.5.1 Data Generated from Morphological/Agronomical and Quality characters

Data based over mean values from each replication were utilized for following statistical analysis of all the characters. The data recorded on different characters were statistically analyzed using software WINDOSTAT version 7.0 developed by Indostat Services Ltd., Hyderabad, India.

1. Analysis of Variance for the Experiment Design

The analysis of variance for Randomized Block Design was carried out on the basis of the model described by Panse and Sukhatme (1985) for individual characters. The statistical model is given below:

$$Y_{ij}=m+a_i+b_j+e_{ij}$$

Where,

Y_{ij} = an observation of the i^{th} genotype in the j^{th} block

M = general mean

a_i = i^{th} genotype effect

b_j = j^{th} replication effect

e_{ij} = random error associated with i^{th} genotype in j^{th} replication

The assumptions of the model are:

a) Observations are independent

b) The random error (e_{ij}) are distributed normally and independently with mean zero and variance σ^2_e

c) The different effects in the model are additive

Analysis of variance tables were constructed for each of the studied traits.

Table 3.5: Skeleton ANOVA

Source	**Degree of freedom**	**Mean squares**	**Expectation of mean square**
Replication	(r-1)	MS_r	$\sigma^2_e+g\sigma^2_r$
Genotype	(g-1)	MS_g	$\sigma^2_e+r\sigma^2_g$
Error	(r-1) (g-1)	MS_e	σ^2_e

Where,

r = Number of replication

g = Number of genotype

MS_r = Mean square due to replication

MS_g = Mean square due to genotype

MS_e = Mean square due to error

σ^2_e = Variance due to error

σ^2_g = Variance due to genotype

σ^2_r = Variance due to replication

2. Computation of Data Recorded

2.1 Mean: The mean value of each character was worked out by dividing the total of corresponding number of observation for each replication.

$$\overline{X} = \frac{\sum X_i}{n}$$

Where,

x_i = any observation in i^{th} genotype

n = number of observations.

2.2 Median:

The was computed by arranging the observations in ascending order of magnitude and the average of the two middle observations was recorded as median.

2.3 Range:

Lowest and highest mean values for each character were recorded for all genotypes.

2.4 Standard Error (S.E.):

It is the dispersion of family mean around the experimental or estimated population mean. Standard error of mean was calculated with the help of error mean square from the analysis of variance.

$$SE\ (d) = \sqrt{\frac{2MS_e}{r}}$$

Where,

MS_e = Mean square due to error

r = Number of replications

2.5 Critical Differences (C.D.):

Critical difference was calculated to compare the treatment means for all the characters. It was computed with the help of S.E. (d) and tabulated value of 't' at 5 percent level of significance at error degree of freedom.

C.D. = S.E. (d) x t

2.6 Coefficient of Variation (Experimental error):

Coefficient of variation was estimated by formula:

$$CV\ (\%) = \frac{\sqrt{\sigma^2_e}}{Mean} \times 100$$

Where,

σ^2_e = error variance

3. Genotypic parameters

The parameters were calculated as given here after:

3.1 Genotypic variance

$$\sigma^2_g = \frac{MS_g - MS_e}{r}$$

3.2 Phenotypic variance:

$\sigma^2_p = \sigma^2_g + MS_e$

Where,

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

MS_e = Mean square due to error/environment

r = Number of replications

3.3 Heritability (Broad sense):

It is the ratio of genotypic variance to phenotypic variance. Heritability in broad sense was calculated by using the formula:

$$h^2_b = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

Where,

h^2 = Heritability in broad sense

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

4. Estimation of Parameters of Variation

The genotypic, phenotypic and environmental coefficients of variations were estimated as below:

4.1 Genotypic coefficient of variation (GCV):

$$GCV = \frac{\sqrt{\sigma^2 g}}{\bar{X}} \times 100$$

4.2 Phenotypic coefficient of variation (PCV):

$$PCV = \frac{\sqrt{\sigma^2 p}}{\bar{X}} \times 100$$

4.3 Environmental coefficient of variation (ECV):

$$ECV = \frac{\sqrt{\sigma^2 e}}{\bar{X}}$$

4.4 Genetic advance (% of mean):

$$G.A\ (\%\ of\ mean) = \frac{K \frac{\sigma^2 g}{\sigma^2 p}}{\bar{X}} \times 100$$

Where,

G.A = Genetic advance

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

K = Selection differential and at 5 percent selection the value of K is 2.06

X = Grand Mean

5. Nature and Extent of Correlation between Different Characters

The nature and extent of correlation between different characters was studied by computing correlation coefficient (r) between pairs of characters. The calculation was based on mean value of characters and the correlation coefficient gives us an idea about the degree of relationship between two traits in direction as well as in magnitude. The correlation coefficients were calculated using the variance and co-variance components (Robinson *et al*., 1951). The correlation coefficients were calculated as follows:

$$r_{xy} = \frac{\sigma_{xy}}{\sqrt{\sigma_x^2 \times \sigma_y^2}}$$

Where,

r_{xy}= Correlation coefficient between character x and y

σ_{xy}= Covariance between characters x and y

σ_x^2 = Variance of characters x

σ_y^2 = Variance of characters y

5.1 Genotypic correlation coefficient (r_g or r_{gxy}):

$$r_{xy} = \frac{\sigma_{gxy}}{\sqrt{\sigma_{gx}^2 \times \sigma_{gy}^2}}$$

Where,

r_{gxy}= genotypic correlation coefficient between variables

σ_{gxy}= Genotypic covariance of the variable x and y

σ_{gx}^2= Genotypic Variance of x variable

σ_{gy}^2= Genotypic variance of y variable

5.2 Phenotypic correlation coefficient (r_p or r_{pxy}):

$$r_{xy} = \frac{\sigma_{pxy}}{\sqrt{\sigma_{px}^2 \times \sigma_{py}^2}}$$

Where,

r_{pxy}= Phenotypic correlation coefficient between variable

σ_{pxy}= Phenotypic covariance of the variable x and y

σ_{px}^2= Phenotypic Variance of x variable

σ_{py}^2= Phenotypic variance of y variable

6. Path-coefficient Analysis

The genotypic coefficients were used to work out path-coefficient analysis. (Dewey and Lu, 1959). A set of simultaneous co-efficients following form were solved in the following algebraic equation:

$r_{ny} = ply + r_{n2}p_{2y} + r_{n3}p_{3y}$ +……+$r_{nx}p_{xy}$

Where,

r_{ny} = Correlation coefficient of one character and yield.

Ply = Path- coefficient between the character and yield.

r_n2, r_n3,……r_{nx} = represents correlation coefficient of that character and each of other yield components in turn.

Path coefficients P_{jy} were obtained as follows:

$p(_{jy})$ = (B^{-1}) x (r)

The indirect effects for a particular character through other characters were obtained by multiplication of direct paths and particular correlation-coefficients between these two characters, respectively.

Indirect effect = r_{ij} x p_{jy}

Where,

i = 1……..n

j = 1……n

r_{ij} = correlation between two independent characters

The residual, *i.e.,* the variation in yield unaccounted for those other associated factors were calculated from the following formulae:

Residual factors (%) = $1-r^2$

Where,

$r^2 = r_{ly}p_{ly} + r_{2y}p_{2y}$ ……+$p_{ny}r_{ny}$

r^2 = is the squared multiple correlation coefficients and the amount of variation in yield that can be accounted for by the yield component characters.

7. Hierarchical Cluster Analysis

Cluster analysis is a method of displaying the similarities and dissimilarities between pairs of genotypes of a set. A commonly used method for forming cluster is hierarchical cluster analysis, using one of the two methods: agglomerative or devisive. In agglomerative hierarchical cluster analysis (used in present study), clusters are formed by grouping cases into bigger and bigger clusters until all cases are members of a single cluster. This procedure attempts to identify relatively homogenous groups of cases (or variables) based on selected characteristics, using an algorithm that starts with each case (variable) in a separate cluster and combines until only one is left.

There are several alternatives available to carryout agglomerative hierarchical cluster analysis. For the present investigation based on morphological and quality

characters data grouping of 20 genotypes of *Z. mays* L was done using Ward's minimum variance method using software WINDOSTAT version 7.0 developed by Indostat Services Ltd., Hyderabad. India. Intra and inter-cluster Euclidean^2 distances generated were used to describe the relationship among the genotypes.

(i) Calculation of Euclidean^2 distance (Genetic divergence)

Euclidean distance is a convenient measure of dissimilarity between individuals (genotypes or population). Euclidean^2 distance [d (ij)] between two individuals I and j, having observations on characters (p) denoted by x_1, x_2, ……,x_p and y_1, y_2…….,y_p for i and j, respectively, can be calculated as under.

$$d(ij) = [(x_1-y_1)^2 + (x_2-y_2)^2 + (x_p-y_p)^2]^{1/2}$$

(ii) Relative contribution of each trait towards genetic divergence

$$C_k = \frac{\sum_{i=I}^{n}(X_{ik} - X_{jk})^2}{\sum_{i=I}^{n}\sum_{k=I}^{c}(X_{ik} - X_{jk})} \times 100$$

Where,

C_k = Percent contribution of k^{th} characters

N = Number of pairs of genotypes i.e. g (g-1)/2, where g is 20 genotypes.

C = Number of characters i.e. 11

X_{ik} & x_{ik} = Standardized mean values of i^{th} and j^{th} member of 1^{th} pair of genotype for k^{th} character

(iii) Group constellation

There is no particular rule of grouping the clusters. Mostly any two genotypes belonging to the same cluster show a small Euclidean^2 values than those belonging to two different clusters. Ward (1963) suggested a technique which was used for grouping various genotypes into different clusters. Ward's method appropriate for quantative variables. This method involves an agglomerative clustering algorithm. It will start out at the leaves and work its way to the trunk, so to speak. It looks for groups of leaves that it forms into branches, the branches into limbs and eventually into the trunk. Ward's method starts out with *n* clusters of size 1 continues until all the observation are included into one cluster.

Based on the notion that clusters of multivariate observation should be approximately elliptical in shape, we assume that the data from each of the clusters will be realized in a multivariate distribution. Therefore, it would follow that they would fall into an elliptical shape when plotted in a *P*-dimensional scatters plot.

Notation used was as follows: Let x_{ijk} denote the value for variable k in observation *j* belonging to cluster *i*.

Furthermore, for this particular method we have to define the following.

Error sum of squares

Here we are summing over all variables, and all the units within each cluster. Here, we are comparing the in individual observations for each variable against the cluster means for that variable. Note that when the Errors Sum of Squares is small, then this suggests that our data are close to their cluster means, implying that we have a cluster of like units.

$$\mathbf{ESS} = \sum_{\mathbf{i}} \sum_{\mathbf{j}} \sum_{\mathbf{k}} |X_{ijk} - X_{ijk}|^2$$

Total Sum of squares

The total sums of squares are defined in the same as always. Here we are comparing the individual observation for each variable against the grand mean for the variable.

$$\mathbf{r^2} = \frac{\mathbf{TSS - ESS}}{\mathbf{TSS}}$$

R-Square

This r^2 value is interpreted as the proportion of variation explained by a particular clustering of the observation.

Using Ward's method we will start out with all sample units in *n* clusters of size 1 each. In the first step of the algorithm, *n*-1 clusters are formed, one of size two and the remaining of size 1. The error sum of squares and r^2 values are then computed. The pair of sample units that yield the smallest error sum of squares, or equivalently, the largest r^2 value will form the first cluster. Then, in the second step 2 of the algorithm, *n*-2 clusters are formed from that *n*-1 clusters defined in step-2. These may include two cluster of size-2, or a single cluster of size 3 including the two items clustered in step 1. Again, the value of r^2 is maximized. Thus, at each step of the algorithm clusters or observations are

combined in such a way as to minimize the results of error from the squares or alternatively maximize the r^2 value. The algorithm stops when all sample units are combined into a single large cluster of size ^{n}. the calculated values can be depicted in two ways one in the form of cluster matrix and another as dendrogram.

3.5.2 Data generated from Biochemical Analysis

For analysis of protein profile data, similarity metrics was computed on the basis of 0/1 binary matrices data where presence of a particular spot with specific R_f value was taken as 1 while its absence as 0. A dendrogram was constructed using unweighted pair group method with arithmetical average (UPGMA) sub programme of NTSYS-pc software.

3.5.3 Data generated from Molecular Analysis

Based on presence/ absence data, genetic similarity was calculated to estimate all pair wise differences in the amplification products for all genotypes. Based on this data, cluster analysis was done to estimate relationship among genotype.

The data generated from polymorphic fragments were analyzed by the equation given by Nei and Li (1979):

$$\text{Similarity} = \frac{2M_X}{M_Y + M_2}$$

Dissimilarity =1-F

Where,

Mx = Number of shared fragments between genotypes y and z

My = Number of scored fragments of genotype y

Mz = Number of scored fragments of genotype z

The 0/1 matrix was used to calculate the similarity genetic distance using 'SIMQUAL' sub-programme of NTSYS-pc software (numerical taxonomy and multivariate analysis system programme) (Rohlf, 1993). Dendrogram was constructed by using distance matrix by the unweighted pair group method with arithmetic average (UPGMA) sub-programme of NTSYS-pc software.

4. RESULTS AND DISCUSSION

Information on genetic diversity and relationship among and between individuals, population, plant varieties and species are important to breeders for the conservation and improvement of crop species. Genetic diversity studies can identify alleles that might affect the ability of the organism to survive in its existing habitat, or might enable it to survive in more diverse habitats (Duran *et al*., 2009). The assessment of the genetic diversity for any crop improvement programme is of prime importance. Various types of markers such as morphological, biochemical and molecular markers are used for this purpose (Barwar *et al*., 2008).

Keeping these facts in view, the present study was carried out in *Zea mays* L., one of the most important coarse cereal crops of India and particularly of Rajasthan so as to enhance its productivity levels matching to international standards. Twenty diverse genotypes were selected which comprised five HQPM (high quality protein maize hybrids), five hybrids, seven composites and three local land races. The crop was raised during *kharif*, 2011 in randomized block design with three replications at Instructional Farm of Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India. Data on 11 morphological/yield characters were recorded. Grain protein was also determined. Seedling of 21 days were used for analyzing biochemical markers *viz*., peroxidase, esterase and superoxide dismutase isozyme. Leaf DNA was extracted for the determination of RAPD and ISSR markers.

Mean data were statistically computed for analysis of variance, genotypic variability, correlation, path analysis and D^2 analysis. Dendogram were constructed as per the recommended standard procedures. SCAR marker determination and designing was also performed. The results obtained are discussed under following heads:

1. Morphological characterization and analysis of variation (ANOVA)
2. Biochemical characterization
3. Molecular characterization, and
4. Development of sequence characterize amplified regions (SCARs) marker

4.1 Morphological Characterization and Analysis of Variance

Success of a breeding programme largely depends on the extent of genetic variability present in the material, greater the diversity in the material better are the

chances for evolving promising and desired types. Phenotypic variability expressed by a genotype or a group of genotypes in any species can be partitioned into genotypic and phenotypic components. The genotypic components being the heritable part of the total variability, its magnitude for yield and related characters influences the selection strategies to be adopted by the breeders.

Morphological markers differ among species, genus and varieties of plants. It is the easiest and quickest way to identify or detect the variation in morphological traits for improvement (Bagali *et al*., 2010). However, these traits are largely affected by environmental variations until and unless these are studied minutely over locations and variable environmental conditions.

4.1.1 Analysis of Variance

The average mean squares for different characters (Table 4.1) revealed that the mean squares due to genotypes were highly significant for all the characters, including grain protein content, indicated the presence of significant genetic variability in the material providing sufficient scope for further selection. Choudhary and Choudhary (2002), Sumathi *et al.* (2005), Salami *et al.* (2007), Turi *et al*. (2007), Akbar *et al.* (2008), Ahmad *et al*. (2011), Idris and Abuali (2011), Atif and Mohammed (2012) also recorded genotypic variations in their material of maize for one or other aforesaid traits.

Table 4.1: ANOVA for various characters in *Z. mays* L.

Characters	Source of variation		
	Replications	**Treatments**	**Error**
Degree of freedom	**2**	**19**	**38**
Leaf number	0.82	1.70**	0.32
Plant height	263.20**	237.16**	31.19
Days of 50% flowering	88.35**	61.24**	6.68
Days of maturity	21.80	186.18**	14.94
Cob length	4.08*	14.29**	1.11
Cob girth	0.27	2.81**	0.23
Grain/ cob	2119.21	34052.91**	2561.28
Grain/row	12.11	93.92**	19.45
Test weight	0.57	41.35**	2.25
Grain yield /plant	144.48**	400.30**	10.20
Biological yield/ plant	57.43	114.65*	61.32
Harvest index	61.14**	98.63**	4.58
Grain protein content	0.42	2.42**	0.54

* Significant at 5 per cent and **Significant at 1 per cent probability level

4.1.2 Mean Performance

Mean value of all characters indicate the normal distribution of genotypes in the present study and hence, representing wide spectrum of variability (Appendix-IV). The coefficient of variation for traits studied being in the range of 2.88 to 13.52 indicated the adequacy of the material and characters studied for further estimation of genetic variability parameter in present investigation (Appendix-IV).

Genotypes classified according to their high *per se* performance for all the characters are listed in Table 4.2. HQPM-5 showed superiority for cob characters *viz*., cob length, cob girth and grain per cob. Genotypes, EQH-63 HQPM-5, HQPM-7 and HQPM-1 exhibited high performance for grain yield per plant. These all line were also superior for grain yield contributing characters thereby indicating that grain yield is the end product of its component.

Table 4.2: Genotypes classified as per their high *per se* performance in *Z. mays* L.

Character	Best Genotypes	Genotypes showing high *per se* performance
Leaf number	PM-6	EQH-63, HQPM-5, B.Sathi, HQPM-1
Plant height(cm)	PM-6	B.Sathi, K.Malan, C.Sathi, Arawali
Days of 50% flowering	HQPM-1	HQPM-5, HQPM-7, EQH-16, EQH-63,
Days of maturity	HQPM-1	HQPM-5, BIO-9637, HQPM-7, EQH-63
Cob length (cm)	HQPM-5	B.Shathi, EQH-63, PHM-1, HQPM-1
Cob girth (cm)	BIO-9637	EQH-63, EC-3161, HQPM-5, PHM-2
Grain/cob	HQPM-5	EQH-63, HQPM-1, EQH-16, HQPM-7
Grain/row	HQPM-5	EQH-16, HQPM-1, Arawali, BIO-9637
Test weight (g)	B.Sathi	BIO-9637, C.Sathi, PM-6, PHEM-2
Grain yield/plant (g)	EQH-63	HQPM-5, HQPM-7, HQPM-1, BIO-9637
Biological yield (g)	PM-6	K.Malan, HQPM-7, EQH-16, Arawali

Harvest index (%)	HQPM-1	HQPM-5, EQH-63, BIO-9637, HQPM-7
Protein content	EQH-63	HQPM-5, BIO-9637, HQPM-1, EQH-16

The total soluble protein of *Z. mays* L. genotypes estimated by Lowry's method is presented in Appendix-II. Grain protein content varied in all the genotypes High soluble protein was recorded in HQPM-5, HQPM-1, EQH-63, HQPM-7, EQH-16 and Navjot over average protein (8.3 mg/g).

Therefore, based on the mean performance, HQPM-5, HQPM-1 and EQH63 displayed superiority for grain yield, biological yield as well as for grain protein content. Therefore, these genotypes could be gainfully utilized in breeding programmes.

4.1.3 Estimates for Genetic Variability Parameters for Various Characters:

Environment has great influence on many quantitative and qualitative traits of plants. This influence showed heritable and non-heritable variation, which can be estimated by the parameters like genotypic coefficient of variation (GCV), heritability and genetic gain.

The genetic parameters were worked out separately for all these characters. The components of variance, heritability, genetic advance as percentage of mean for various characters are presented in Table 4.3. It revealed that phenotypic coefficient of variation (PCV) was higher than respective genotypic coefficient of variation (GCV), but a relatively marginal difference was been observed between PCV and GCV for leaf number, plant height, days to maturity, cob girth, grains yield per plant, harvest index and grain protein content revealed that variability was due to genotypic differences. On the other hand, environmental influences were predominant for the characters like days of 50% flowering, cob length, grain per cob, grain per row, test weight and biological yield per plant. Therefore, selection based on the above characters is expected to be effective while for other characters selection must be performed carefully considering environmental factors.

High GCV was recorded for test weight, grains per cob, grains per row and harvest index. Therefore, selection for all these characters would be effective. Similar results were also reported by Kabdal *et al.* (2003) for grain yield, ear length and harvest index in *Z. mays* L. genotypes. Moderate GCV were recorded for grain yield per plant, cob length, cob girth, days to 50% flowering, leaf number and days to maturity. However characters like plant height and biological yield per plant showed comparatively low estimates of GCV and PCV indicating that these characters were highly influenced by the environment.

Estimates of GCV and PCV alone do not help in assessing the amount of heritable variation. Genetic contribution to phenotypic expression of a trait is better reflected by the estimates of heritability. High estimates of heritability indicate presence of more fixable variability (Shivakumar *et al.*, 2011). In the present study, heritability (broad sense) estimates were high (>70%) for all the traits except leaf number, plant height, grains per row, biological yield and grain protein content. The estimates of heritability in broad sense were moderate to high for most of the characters *viz*., grains per cob, test weight, grain yield per plant and harvest index. Mahmood *et al.* (2004) reported broad-sense heritability, for days taken to tasseling, number of days taken to silking, plant height, ear length, number of kernel rows per ear, number of kernels per row, 100-grain weight and grain yield per plant in *Z. mays.* Vashistha *et al*. (2013) revealed high broad-sense heritability were observed for plant height, ear height and ear girth in maize. High to moderate heritability with moderate estimates of genetic advance recorded for biological yield, grain yield per plant, plant height and ear height where careful selection may lead towards improvement for these traits.

Burton (1952) suggested that GCV along with heritability estimates would give a better idea about the efficiency of selection as it simply depicts the amount of genetic variation while heritability measure the proportion to which the variability of a character is transmitted to its progenies. However, Johanson *et al.* (1995) suggested that variability and genetic advance when calculated together would more useful in predicating the resultant effect of selection on phenotypic expression.

While assessing the overall position, the present study revealed high genetic advance as percentage of mean (genetic gain) along with estimate of heritability and GCV for test weight, grain yield per plant, grains per cob, cob length, grains per row and harvest index. Kabdal *et al.* (2003) reported high heritability and genetic advance for grain yield, ear height, plant height and ear length. However, high heritability with moderate genetic advance was recorded for days to 50% flowering, cob length, cob girth, grain yield per plant and days to maturity indicating involvement of both additive and non-additive gene action and hence selection for these characters based on phenotypic observations alone may not be effective. Bello *et al*. (2012) revealed high heritability along with high genetic advance recorded for grain yield, grains per ear, ear weight, plant and ear heights providing evidence that these parameters were under the control of additive gene effects and effective selection could be possible for improvement of these characters.

4.1.4 Correlation Coefficient Analysis:

The knowledge of genetic correlation for seed yield, its components and various quality characters become very important with the problems of combining high yield potentials with desirable agronomical and qualitative parameters. Hence association studies would provide reliable information on nature, extent and direction of selection for any crop improvement programme.

The *inter se* correlation coefficients at phenotypic and genotypic levels between different characters for grain yield are depicted in Table 4.4. In majority of cases, the coefficient for genotypic correlation exhibited its higher magnitude over respective phenotypic correlation coefficient indicating the strong inherent nature of characters studied.

Grain yield per plant showed significant positive genotypic and phenotypic correlation with days of 50% flowering, days of maturity, cob length, grains per cob, cob girth, grains per row and harvest index. Whereas significant positive genotypic correlation was recorded for leaf number. Similarly, Sumathi *et al.* (2005) studied genotypic correlation and showed that ear weight, number of rows per ear, number of kernels per row, and total number of kernels per ear was positively associated with grain

yield. Wali *et al.* (2006) observed that grain yield was positively associated at genotypic and phenotypic level with plant height, ear length, ear circumference, number of kernels per row, fodder yield per plot and 100-grain weight, but was negatively correlated with number of days to 50 per cent silking at the phenotypic and genetic levels. Heping *et al*, (2006) noticed that grain yield was significantly correlated with plant height, ear diameter, ear length, rare ear length, 100-kernel weight and grain production rate. Grain yield was most highly correlated with ear diameter, followed by 100-kernel weight, plant height, ear length and grain production rate. Saidaiah *et al*. (2008) also reported that grain yield per plant had significant and positive relationship with plant height, ear height, number of leaves, flag leaf area, chlorophyll content at 50 % silking, ear length, ear girth and 100 seed weight.

Harvest index showed positive and significant correlation with days of 50% flowering, days of maturity, cob length, grains per cob, cob girth and grains per row at both genotypic and phenotypic level but significant negative correlation with biological yield. Vasic *et al.* (2001) reported positive and highly significant correlation of harvest index with plant height, ears plant-^{1}and grain yield. Whereas, Inamullah *et al*., (2011) reported positive and significant correlation of harvest index with grain yield and biological yield.

Biological yield shows negative correlation with cob length, cob girth and test weight, while significant positive correlation was not observed. Test weight showed significant positive correlation with cob girth and plant height and negative correlation with grains per cob, days of 50 percent flowering and days of maturity. Earlier Saidaiah *et al*. (2008) also reported significant positive correlation of 100 seed weight with plant height, number of leaves above ear, flag leaf area, chlorophyll content at 50 per cent silking, ear length and ear girth.

Grains per row presented significant positive correlation with days to 50 percent flowering, days to maturity, cob length, cob girth and grains per cob at phenotypic and genotypic level. While, negative genotypic correlation was estimated with plant height. Grains per cob showed significant positive correlation with leaf number, days to 50 percent flowering, days to maturity, cob length and cob girth at genotypic and phenotypic

level. A significant positive correlation existed for cob girth with days to 50 percent flowering, days to maturity and cob length at genotypic and phenotypic level respectively.

Cob length and days to maturity presented significant positive correlation with days to 50 percent flowering. A negative correlation existed for days to 50 percent flowering with plant height. At genotypic and phenotypic positive significant correlation of plant height was observed with leaf number. Hema *et al.* (2001) and Saidaiah *et al*. (2008) had also reported negative correlation between yield and anthesis silking interval.

4.1.5 Path Coefficient Analysis:

The path coefficient analysis developed by Wright (1921), which measures the direct influence of one variable upon the other, and permits separation of correlation coefficients into components of direct and indirect effects. The direct and indirect effects provide actual information on contribution of characters and thus form the basis for selection to improve the yield.

In the present study path coefficients were analyzed for all the 11 characters on grain yield per plant using genotypic as well as phenotypic correlations. As observed from correlation study Table 4.4, grain yield per plant exhibited strong positive with days of 50% flowering, days of maturity, cob length, grains per cob, cob girth, grains per row and harvest index. However, path analysis (Table 4.5) revealed direct and indirect contribution of component characters on grain yield per plant. Maximum positive direct effect on grain yield were recorded by harvest index followed by biological yield, grains per cob and grains per row at genotypic and phenotypic level. Ei-Shouny *et al*. (2005) also reported direct contribution of test weight followed by grains per row, grain rows per ear, ear length and ear diameter and ear diameter on grain yield. Saidaiah *et al*. (2008) reported positive direct effect of plant height, leaves above ear and 100-seed weight towards grain yield.

Harvest index exhibited highest positive direct effect and significant correlation coefficient on grain yield per plants, indicating strong relationship, hence direct selection could be effective for yield improvement. The direct effect of biological yield was positive, however, the correlation coefficient was negative but indirectly it affects yield

therefore, selection for this character appeared to be important for yield improvement. However, direct effect and correlation coefficient was significantly positive for grains per cob and grains per row with grain yield per plant. Which might be due to the indirect positive effect of biological yield and harvest index. Further, low estimates of residual path way indicate that the characters included in the study mainly contributed towards grain yield and remaining characters were of less importance.

Based on path analysis the most important characters identified as contributing traits included harvest index, biological yield followed by grains per rows. Which could be gainfully utilized for improvement.

Results obtained from the present study based on genetic variability parameters, correlation and path analysis reveled that the selection for harvest index, biological yield and grains per row could enhance the productivity level of *Z. mays* L. Based on mean performance, genotypes HQPM-5, HQPM-1, EQH-63, and HQPM-7 were identified as superior for most important yield and quality characters.

4.1.6 Genetic Divergence Analysis:

For developing improved varieties, exploitation of available genetic diversity is one of the most important criteria which help in an effective breeding programme. Multivariate analysis is a potent biometrical tool for quantifying the degree of divergence in the germplasm (Maloo and Bhattacharjee, 1999). To achieve breakthrough in the yield and quality characters of *Z. mays* L. genetic divergence analysis was attempted so that the highly diverse genotypes could be selected for molecular breeding programmes.

The genetic divergence analysis was done for all the 11 characters studied in the present study. Hierarchical cluster analysis of 20 genotypes into five clusters at 100 Mahalnobis Euclidean^2 Distance by Ward's minimum variance dendrogram (Figure 4.1) with variable number of genotypes which indicated the presence of considerable amount of genetic diversity in the material (Table 4.6).

Table 4.6: Cluster profile of 20 genotype of *Z. mays* L.

Cluster	No. of Genotype	Genotypes

I	4	HQPM-1, HQPM-5, HQPM-7, EQH-63
II	6	EQH-16, PM-4, PHM-2, BIO-9637, PM-5, PM-6
III	5	Navjot, PM-3, Arawali, K. Malan, EC-3161,
IV	2	PHM-1, HM-8
V	3	B.Sathi, C.Sathi, PHEM-2

Cluster II was the largest contained 6 genotypes followed by cluster III which possessed 5 genotypes. The I, V and IV possessed 4, 3 and 2 genotypes, respectively. Each cluster has different types of genotypes irrespective to their nature of genetic make up belonging to hybrids, composities, land races etc.

The distribution pattern of genotypes of diverse origin in a single cluster indicated that genetic diversity observed within *Z. mays* L. The genetic stock was not related to their geographical origin. Noted differences in plant characters probably occurred over time due to the migration of alleles among genetic stocks, cross-pollinated or spontaneous mutation and/or free movement of plant materials from one location to another.

a) Intra and inter cluster distances:

The intra- and inter cluster distance values between 5 clusters are presented in Table 4.7 and also Euclidean^2 Distance in Figure 4.2. The perusal of the Table 4.7 revealed that inter-cluster distances were greater than intra-cluster distances, revealing considerable amount of genetic diversity among the genotypes studied. Genotypes belonging to clusters with maximum intra-cluster distance are genetically more divergent and hybridization between divergent clusters is likely to produce wide variability with desirable segregant (Maloo and Bhattacharjee, 1999). The average intra-cluster distance between the genotypes was maximum (30.92) for the cluster III followed in descending order by cluster I (15.91), II (12.12), IV (0.0) and V (0.0) respectively. The highest inter-cluster distance was noted between cluster I and III (91.55) followed by cluster III and V (70.31), III and IV (49.84), IV and V (43.90) and so on. The least (22.12) inter-cluster distance was recorded for cluster I and IV.

b) Mean values of different clusters for various characters:

Cluster mean and general mean value for 11 characters for all the 20 *Z. mays* L. genotypes are presented in Table 4.8 which revealed that differences in cluster means existed for all the characters studied. The cluster I comprising 4 genotypes was characterized as having above average values for leaf number, days to 50 percent flowering, days to maturity, cob length, cob girth, grain per cob, grain per row, grain yield per plant and harvest index. Cluster II comprised 6 genotypes and were characterized for plant height, days to 50 percent flowering, days to maturity, cob girth, grains per cob, test weight, grain yield per plant, biological yield and harvest index in particular being above average in this cluster. Cluster III involving 5 genotypes were characterized for leaf number, plant height, grain per cob and biological yield.

Cluster IV had 2 genotypes and above average values for cob length, grain per row and biological value. Similarly, cluster V comprising of 3 genotypes for leaf number, plant height, cob length, test weight, grain yield per plant and harvest index. The percent contribution towards total genetic divergence was maximum for the characters like test weight, harvest index, grains per row and plant height as also reported by Singh *et al*. (2005), More *et al*. (2006) and Ganesan *et al*. (2010). The genotypes HQPM-1, HQPM-5, HQPM-7 and EQH-63 were showing maximum divergence at above said characters. These genetically diverse genotypes could be further used for developing superior hybrids and can also be utilized in developing synthetics and composites.

Table 4.7: Estimates of intra (diagonal) and inter-cluster distances in 20 genotypes of *Z. mays* L.

Cluster	**I**	**II**	**III**	**IV**	**V**
I	**15.91**	41.89	35.04	22.12	41.14
II		**12.12**	91.55	41.33	32.01
III			**30.92**	49.84	70.31
IV				**00.00**	43.90
V					**0.00**

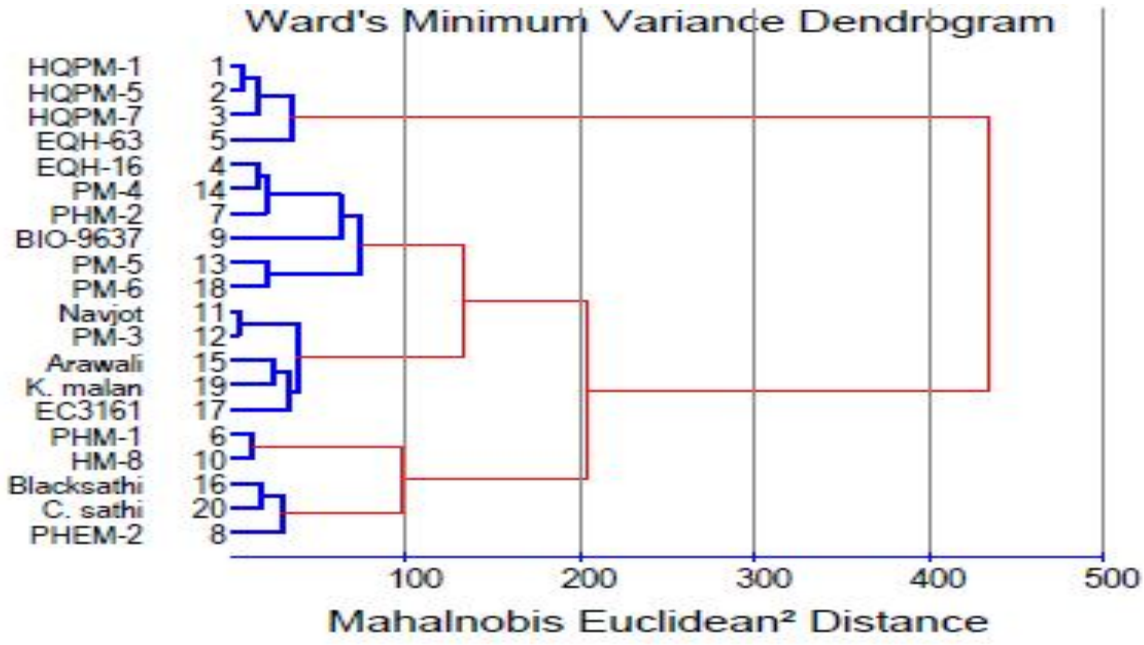


Figure 4.1: Ward's Minimum Variance Dandrogram for 20 genotype of *Z. mays* L.

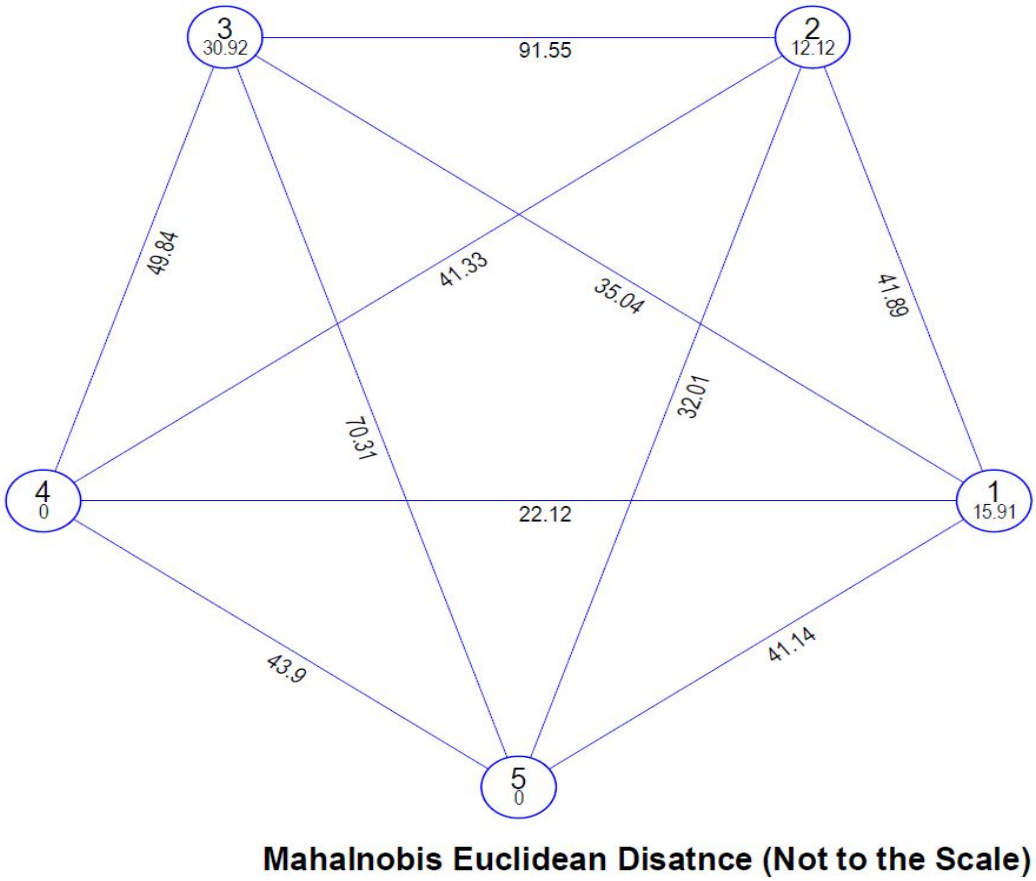


Figure 4.2 : Intra and inter cluster distance for 7 groups of 20 genotypes of *Z. mays* L.

4.2 Biochemical Characterization

Cellular differentiation, morphological development, and functional specialization of an organism are related to changes in biochemical characters including isozyme patterns. Isozyme refers to multiple molecular form of an enzyme sharing catalytic activity derived from a tissue of single organism (Market and Moller, 1959). Isozymes have been used as biochemical markers in plant and animal breeding (Edwards *et al*., 1987 and Geldermann, 1975).

Isozymes are the products of genes through transcription and translation processes, therefore their expressions depend on the stage of plant growth as well as plant tissues. Being gene products, isozyme show band intensity that is proportional to the dosage of the encoding gene (Abdullah, 2001). The difference in isozyme patterns are usually directly related to organism's metabolic activity. Therefore, results of isozyme analysis cannot only estimate the genetic structure of a population, but can also be used to compare relevant performance traits with specific isozyme among individuals or stocks in genetic improvements programs (Sharma *et al*., 2008).

The isozymes analyses have several advantages compared to morphological markers. The alleles (allozyme) at most loci are co-dominant. This co-dominance causes no deleterious changes in plant phenotype through recessiveness or pleotrophy and allows heterozygous to be distinguished from homozygous. Any plant tissue or material can be used as sample including leaves, roots, pollen, and callus. It is also possible to screen plant at seedling stage and retains only desirable genotypes. Isozyme analysis is widely used for its relative efficiency and cost effectiveness, particularly in studies of intraspecific variability (Smila *et al*., 2007 and Johnson *et al*., 2010).

In the present study, diversity at biochemical level in 20 maize lines was analyzed through isozymes *viz*., esterase, peroxidase and superoxide dismutase.

4.2.1 Esterase (EST; E.C.3.1.1.1)

An esterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water called hydrolysis. Esterase hydrolyzes toxic compounds like pesticides from plant can serve as a detoxification or activation mechanism that can govern pesticide selectivity or resistance, and initiate or determine the rate of pesticide biodegradation in the environment.

Esterase isozymes exhibited a maximum of two bands having Rm value of 0.1 and 2.5 (Fig. 4.3). The enzyme banding pattern showed that band number 1 and 2 (Rm= 0.1 and 0.25) were present in all genotypes. The differences were observed in terms of intensity of bands and size of bands reflecting genotypic variability. EQH-16, EQH-63, Bio-9637, HM-8, PM-3 and PM-4 exhibited high intensity (Plate-2). High intensity bands of each genotype were more towards cathodic side, possibly having net positive charge and high molecular weight, while rest of the bands were towards anode indicating net negative charge and corresponding low molecular weight. High *per se* performance for grain protein content, days to 50 percent flowering, days to maturity, cob girth, grain per row, grain per cob, grain per yield and harvest index. Polymorphism in esterase isozyme was reported in different species of maize in vitro by Rao *et al.* (1997) and they speculated that esterase banding pattern could be used as a biochemical marker for the identification of species in maize.

4.2.2 Peroxidase (PER; E.C.1.11.1.7)

Peroxidase oxidizes a vast array of compounds (Hydrogen donors) in the presence of hydrogen peroxide (H_2O_2). Plant peroxidases are heme-containing glycoproteins and are usually classified as acidic, neutral or basic, according to their isoelectric points. Higher plants possess a large number of peroxidase isozymes, which are encoded by multigene families. Several physiological functions for peroxidases in plants have been reported, such as removal of H_2O_2, oxidation of toxic reductants,biosynthesis and degradation of lignin in cell walls (Grisebach, 1981; Mader and Fussl, 1982; Lagrimini,1991), auxin catabolism (Hinnman and Lang, 1965; Gazaryan and Lagrimini, 1996), defensive responses to wounding (Espelie *et al*., 1986; Dowd and Lagrimini, 1997) as well as defense against pathogen and insect attack (Ye *et al*., 1990; Dowd and Lagrimini, 1997).

Peroxidase isozyme was widely used for assessment of genetic diversity in various crops like peanut (O'Donell *et al*., 1992), wheat germ (Converso and Fernandez, 1995), rice (Srivastva *et al*., 2002), sugarcane (Manjunatha *et al.*, 2003) and pearl millet (Smila *et al*., 2007).

In the present study, electrophoretic profiles of peroxidase isozyme showed two activity zones having Rm value of 0.25 and 0.44 (Fig. 4.4). Both bands were present in all genotypes (Plate-3). Hannan and Orick (2000) while studying the genetic variation between two *Iris* species on the basis of isozyme analysis found that the presence of similar isozyme patterns is the evidence for the presence of geologically single origin of both species from a single pool.

4.2.3 Superoxide Dismutase (SOD; E.C. 1.15.1.1)

Within a cell, the superoxide dismutase (SODs) constitutes the first line of defense against reactive oxygen species (ROS) and present in all subcellular locations (Alscher *et al*., 2002). Superoxide dismutase also plays an important role in assessment of genetic variability as a biochemical marker. Isozyme profile as observed for SOD for *Z. mays* L. genotypes is presented in Plate-4. Corresponding SOD zymogram indicated four bands having Rm value 0.1, 0.29, 0.41 and 0.55 present in all genotypes (Fig. 4.5). In addition, there were genotypes that could be differentiated by variable intensities (quantative differences) as well as banding patterns (qualitative differences). Navjot, B.Sathi and PM-6 showed banding pattern at Rm 0.1, but these genotypes were genetically diverse and belong to variable geographical origin. While, at Rm 0.29 all genotypes showed banding pattern with high intensity and more size. PHM-1, PHEM-2, PHM-2, Bio-9637, HM-8, Arawali and PM-3 showed high intensity band and rest with less intensity at Rm 0.41. These genotypes also exhibited wide range of morphological variations in terms of their phenotypic constitution and were also clubbed into different clusters based over D^2 analysis. Whereas, single unique banding pattern was seen in all genotype at Rm 0.55. Similarly, Patra and Chawala (2010) showed unique banding pattern in basmati rice, wherein, they analysed five isozymes *viz.* alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), esterase (EST), peroxidase (POX) and superoxide

dismutase (SOD) by Native-PAGE. They reported that SOD revealed monomorphic banding pattern in basmati rice.

Thus, in the present study based on biochemical characterization using three isozymes *viz.*, esterase, peroxidase and superoxide dismutase exhibited presence of 8 diverse alleles in all. Only superoxide dismutase isozyme showed 63% polymorphism, which could be attributed to environmental condition. Isozyme similarity matrices of 20 genotypes of *Z. mays* L. revealed the relationship between different genotype ranged from 0.88 to 1.00. Similarly to present work, Aboel-Atta and Ahmed (2009) studied three isozyme, α- and β-esterase and aldehyde oxidase, out of which only the banding patterns, α- and β-esterase revealed slight polymorphism with a percentage of 27.77% between the two studied species of *Melilotous.*

Cluster tree analysis was carried out by UPGMA method based on genetic distance. In all genotypes were divided into two clusters at a similarity coefficient of 0.88. Cluster-I include sixteen genotypes *i.e.* HQPM-1, PHM-1, Arawali, HQPM-5, PHEM-2, PM-3, PM-6, HQPM-7, PHM-2, K.Malan, EQH-16, BIO-9637, EC-3161, EQH-63, HM-8 and C.Sathi at similarity coefficient of 1.00. Cluster-II included PM-5, PM-4, Navjot and B.Sathi with similarity coefficient of 1.00. Therefore, genetic diversity and distance as derived from isozyme analysis showed limited polymorphism as also reported by Sonnante *et al*. (1997) in *Vigna* species. Among all the genotypes PM-3, Bio-9637 and HM-8 appeared to be more diverse at biochemical level which appears to be showing genetic variations at morphological level also.

4.3 Molecular Characterization

Germplasm characterization and evolutionary process in viable population are important links between the conservation and utilization of plant genetic resources. The development of molecular and biochemical techniques help researchers not only to identify genotypes, but also in assessing and exploiting the genetic variability (Whitkus e*t al.*, 1994). The isozymes represent allelic expression of the same locus, but fragments produced by RAPD are independent genetic markers (Ochiai *et al*., 2001) with a lower proportion of non-neutral markers than formerly (Bartish *et al*., 2000). But in comparison to molecular markers isozyme markers are very limited and also are influenced by

environment as also highlighted by Horacek *et al*. (2009). Germplasm characterization using molecular markers may contribute to knowledge of genetic relationships between accessions of wild and cultivated gene pool more precisely and hence facilitate the breeding of genotypes to satisfy the market needs and respond to diverse biotic and abiotic challenges. Thus, DNA markers are more reliable because the genetic information is unique for each species and is independent of age, physiological conditions and environmental factors (Kalpana *et al*., 2004). The molecular marker technique efficiency is based on the amount of polymorphism it can detect in the given accessions (Leela *et al*., 2009).

Significant progress has been made in recent years in the application of molecular markers to plant genetic resources characterization and evaluation (Soltis *et al*., 1992). Among several efficient methods for revealing genetic variability within and among plant populations, some of the most widely applied methods are RAPD (Wollf *et al*., 1993; Brummer *et al*., 1995; Wachira *et al*., 1995; Swoboda and Bhalla, 1997) and ISSR (Guo *et al*., 2006; Joshi and Dhawan, 2007; Heikal *et al*., 2008a). Many researchers (Garkava *et al*., 2000; Matos *et al*., 2001; Ochiai *et al*., 2001) pointed out that DNA-based markers were superior to isozyme in detecting genetic diversity. Therefore, RAPD and isozyme analysis often give discordant patterns suggesting the importance of using multiple molecular marker system in studies of population structure (Wendel and Doyle 1998; Bartish *et al*., 2008; Lebot *et al*., 2003). Further particularly RAPD and ISSR are simpler to use then simple sequence repeats (SSR) technique as prior knowledge of target sequences of flanking of the repeat regions is not required (Reddy *et al*., 2002).

4.3.1 DNA Isolation, Purification and Quantification

The problems encountered in the isolation and purification of DNA especially from mechanical and aromatic plants include degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, inhibitors compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Padmalata and Prasad, 2006). Moreover, the contaminating RNA that precipitates along with the DNA causes many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993), interference with DNA amplification

involving random primers, e.g. RAPD analysis (Mejjad *et al.*, 1994), and improper priming of DNA templates during thermal cycles sequencing. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR condition is required.

Tannins, terpens and resins considered as secondary metabolites are also difficult to separate from DNA (Ziegenhagen and Schilz, 1998). Certain polysaccharides are known to inhibit RAPD reactions. They disort the results in many analytical applications and therefore lead to wrong interpretations (Kotchoni *et al*., 2003). Polysaccharides like contaminants, which are undetectable by most criteria, can cause anomalous reassociation kinetics. Polysaccharide co-precipitation is avoided by adding a selective precipitant of nucleic acid, i.e. CTAB (2%) to keep polysaccharides in solution. Many DNA isolation procedures also yield large amount of RNA. (Mejjad *et al*., 1994). Large amount of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR. A prolonged overnight RNase treatment degraded RNA into small ribonucleosides that do not contaminate the DNA preparation, and yielded RNA free pure DNA.

Quantity and quality of the DNA samples was determining absorbance at 260 nm and 280 nm using UV-spectrophotometer, DNA concentrations were also rechecked by visual assessment of band intensity known concentration (50 ng/µl) using 0.8% agarose gel.

The amount of DNA isolated from various genotypes of *Z. mays* L. ranged from 1060 to 3685 µg/ml (Table 4.9).

Table 4.9: Quality and quantity of total genomic DNA of *Z. mays* L.

S.No.	Genotype code	Genotype	Quality (A260/A280)	Quantity (µg/ml)
1	G_1	HQPM-1	1.87	1230
2	G_2	HQPM-5	1. 89	1690
3	G_3	HQPM-7	1.78	1060
4	G_4	EQH-16	1.82	2590
5	G_5	EQH-63	1.84	3685
6	G_6	PHM-1	1.81	3226
7	G_7	PHEM-2	1.78	2345

8	G_8	PHM-2	1.80	3290
9	G_9	BIO-9637	1.83	1567
10	G_{10}	HM-8	1.70	1780
11	G_{11}	Arawali	1.81	1905
12	G_{12}	PM-3	1.84	2354
13	G_{13}	PM-4	1.82	2469
14	G_{14}	PM-5	1.80	2890
15	G_{15}	Navjot	1.78	2780
16	G_{16}	B.Sathi	1.84	1569
17	G_{17}	PM-6	1.82	2460
18	G_{18}	K.Malan	1.80	3120
19	G_{19}	EC-3161	1.84	2346
20	G_{20}	C.Sathi	1.80	2570

A ratio of absorbance at 260 nm and 280 nm (A260/A280) which ranged from 1.70 to 1.89 showed that the DNA was free from contaminants like polysaccharides, protein and RNA. The quality of DNA was also checked by gel electrophoresis (0.8%) that revealed a single discrete band in all genotypes (Plate-5) showing that genomic DNA was intact and had high molecular weight, free from any mechanical or enzymatic degradation, free from RNA contamination and was of high quality.

4.3.2 Molecular marker analysis

Genetic diversity assessment can increase the effectiveness of breeding programms (Fan *et al*., 2006). Among the various DNA marker-assisted techniques available, the randomly amplified polymorphic DNA (RAPD) technique (Williams *et al*., 1990) has been most popular because of speed, low cost and the use of only minute amount of plant material for analysis. Similarly, ISSR markers are useful in detecting polymorphism among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome (Reddy *et al*., 2002).

RAPD and ISSR marker are being successfully used in genetic diversity analysis of various crops (Singh *et al*., 2009 and Behera *et al*., 2008). In the present study, a total

of 20 random decamer primers (RAPD) and 15 inter simple sequence repeats (ISSR) primers were used for RAPD and ISSR analysis, respectively, to generate DNA fingerprint profile of 20 *Z. mays* L. genotypes with a view to detect polymorphism and access genetic diversity among genotypes.

4.3.2.1 Optimization of PCR conditions for RAPD and ISSR:

PCR amplification conditions *viz.*, concentration of template DNA, primers, $MgCl_2$, Taq DNA polymerase and annealing temperature were optimized for RAPD and ISSR primers. Different temperature and time intervals during denaturation, annealing and elongation steps were also optimized which affect amplification, banding and reproducibility. Genomic DNA concentration varying from 15 to 100 ng, primer 0.2, µM to 1 µM, $MgCl_2$, 1.0 mM to 2.5 mM and Taq DNA polymerase 0.5 U to 1.5 U were used for PCR amplification. Different annealing temperatures *viz.* 35^0C, 36^0C, 37^0C, 38^0C, 39^0C and 40^0C were used during PCR amplification for RAPD primers. In general, inconsistent bands were obtained at annealing temperatures of 35^0C and 36^0C.

In brief, reproducible and clear banding pattern were obtained in a reaction mixture of 20 µl containing 25 ng of template DNA, 2 µl of 10 x Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 0.30 µM of primer and 1 unit of Taq DNA polymerase. The annealing temperature of 36^0C was found to be optimum for generating clear and reproducible bands for RAPD primers. For ISSR primers, annealing temperature varied from 22^0 to 53^0 for PCR amplification.

4.3.2.2 Polymorphism in *Z. mays* L. using RAPD Primers:

Twenty RAPD primers having 60% or more GC content were used for the present investigation. Out of these, 16 showed amplification to the extent of 73.41% polymorphism. A total 79 amplified bands were obtained, out of which 58 were polymorphic.

Table 4.10: DNA amplification profile and polymorphism generated in *Z. mays* L. using 16 RAPD primers

S.No.	Primer code	MW (bp)	Total no of bands	No. of polymorphic bands

1	OPA-01	350-1400	6	5
2	OPC-08	800-2000	2	1
3	OPD-05	180-1300	9	9
4	OPD-12	450-1500	4	4
5	OPE-03	400-1600	5	4
6	OPF-17	400-1200	2	2
7	OPJ-04	500-1600	6	3
8	OPP-01	200-900	7	5
9	OPP-02	400-1200	4	3
10	OPP-03	400-900	2	1
11	OPP-04	650-1000	2	1
12	OPP-05	500-1000	4	4
13	OPP-07	300-1500	6	3
14	OPP-10	200-2000	8	8
15	OPP-12	400-1500	5	2
16	OPP-16	450-2500	7	3
Total			79	58

The DNA amplicon size and polymorphism generated among various genotypes of *Z. mays* L. using RAPD primers are presented in Table 4.10. The total number of bands observed for every primer were recorded separately and polymorphic bands were checked subsequently. The total number of amplified bands varied between 2 (primer OPC-08, OPP-04, OPF-17 and OPP-03) and 9 (primer OPD-05) with an average of 4.9 bands per primer. The polymorphism of all 20 genotypes *Z. mays* L. were 73.41% and the overall size of PCR amplified products ranged between 180 bp to 2500 bp. Similar to present findings Mukharib *et al.* (2010) also obtained high level of polymorphism of 73.02 per cent among maize inbred lines. Earlier, Bruel *et al.* (2007) used RAPD molecular markers to analyze genetic diversity between 16 corn lines. Twenty-two primers were used resulting in the amplification of 265 fragments, of which 237 (84.44%) were polymorphic.

DNA banding profile of individual plant DNA samples from 20 genotypes of *Z. mays* L. after amplification with RAPD primers are depicted in Plate 6 to 14.

4.3.2.3 Genetic Relationship and Cluster Tree Analysis on RAPD data:

The data obtain using RAPD (Appendix-IV) were further used to construct similarity matrix of *Z. mays* L. genotypes using 'Simqual' sub-programme of software NTSYS-pc. Dandrogram was constructed using similarity matrix values as determined from RAPD and ISSR data for 20 *Z. mays* L. genotypes using unweighted pair group method with arithmetic average (UPGMA) sub-programme of NTSYS-pc software.

Based on RAPD similarity matrix data (Table 4.11), the value of similarity coefficient ranged from 0.34 to 0.76 i.e. 34-76% or. The average similarity across all the genotypes was found out to be 0.63 showing that genotypes were polymorphic. Maximum similarity value of 0.76 was observed between genotypes PM-6 and B. Sathi. These genotypes showed high *per se* performance for leaf number and plant height for morphological traits as well as biochemical level. Similarly, minimum similarity value of 0.34 was observed between genotypes Arawali and HQPM-5 and these genotypes were more diverse for plant height and biological yield and at biochemical level. Similarly, Valdemar *et al.* (2004) used 81 maize accessions to analyze genetic diversity using RAPD markers. Thirty-two highly informative primers amplified 255 markers of which 184 (72.2%) were polymorphic. Mukharib *et al*. (2010) analyzed eight maize inbred lines for assessment of genetic diversity using RAPD markers. They detected high level of polymorphism of 73.02 per cent among the genotypes. The maximum genetic distance of 29.7 per cent was detected between CM-202 and KDMI-16, while, the minimum genetic distance of 12.8 per cent was observed between KDMI-04 and CI-05.

4.3.2.4 Cluster Tree Analysis

The RAPD cluster tree analysis of 20 maize genotypes showed that they were mainly divided into main two clusters at a similarity coefficient of 0.45 (Fig 4.6). Genotypes HQPM-5 and EQH-16 were out-grouped from all other genotypes at a similarity coefficient of 0.45 and formed the first cluster. At a coefficient of 0.52 was the second cluster having all remaining genotypes. Genotypes PM-3 and C.Sathi were again out-grouped and formed another solitary cluster at a similarity coefficient of 0.52. The second subcluster of cluster II was further divided into two main clusters at a similarity coefficient of 0.58 and out grouped genotype EC-3161, from all other genotypes. The

subcluster of cluster II was again divided into major cluster at a coefficient of 0.59. Here, first cluster contained a single genotype Arawali, and second cluster being the major cluster, and further got separated into two minor groups at a coefficient at 0.64. Minor I group contained 10 genotypes *viz*. PHEM-2, PHM-2, Bio-9637, HM-8, B.Sathi, PM-6, K.Malan, Navjot, PM-4 and PM-5, while group II had only four genotypes *viz.*, HQPM-1, HQPM-7, EQH-63 and PHM-1 (Fig 4.6).

The cluster tree revealed similar results based over similarity matrices. The association amongst different genotypes presented in the form of dendrogram, the genotypes which lay nearer to each other in dendrogram were more similar to one another than those lying apart. The dendrogram (Fig. 4.6) also showed the relative magnitude of resemblance among different genotypes used in present investigation.

4.3.2.5 Principal Component Analysis (PCA):

Two and three dimensional principal component analysis based on RAPD data (Fig. 4.7 and 4.8 respectively) showed similar clustering pattern of 20 genotypes as evident from cluster tree analysis. Most of the genotypes were in one major cluster that included EQH-63, PM-6, Bio-9637, PHM-2, Navjot, HQPM-1, HQPM-7, PM-5, K.Malan, PHEM-2, B.Sathi and PHM-1. The two minor clusters included PM-3 and C. Sathi in one and Arawali and EC-3161 in another.

4.3.2.6 Polymorphism in *Z. mays* L. using ISSR Primers:

Fifteen ISSR primers were used for the present investigation, out of which ten primers showed amplification in all genotypes except five, *viz.*, primer-802, primer-804, primer-806, primer-807 and primer-810. The 10 ISSR primers amplified, total 59 bands of which 51 were polymorphic. The total number of bands observed for every primer was recorded separately and polymorphic bands percentage was calculated subsequently (Table 4.12). The total number of amplified bands varied between 2 (primer-808) and 8 (primer-852) with an average 5.9 per primer.

Table 4.12: DNA amplification profile and polymorphism generated in *Z. mays* L. using 10 ISSR primers

S.No.	Primer code	MW (bp)	Total no. of bands	No. of polymorphic bands

1	Primer-851	30-1150	6	4
2	Primer-852	350-1200	8	8
3	Primer-854	300-1150	7	4
4	Primer-803	250-1350	8	8
5	Primer-805	300-1150	5	4
6	Primer-808	100-1200	2	2
7	Primer-853	300-1150	4	2
8	Primer-855	400-1600	8	8
9	Primer-857	250-1100	7	7
10	Primer-856	500-2200	4	4
Total			59	51

The polymorphism percentage ranged from as low as 50% (primer-853) to as high as 100 % in six primers (Primer-852, Primer-803, Primer-808, Primer-855, Primer-857, Primer-856). Average polymorphism across all the 20 genotypes of *Z. mays* L. was found to be 86.44%. Overall size of PCR amplified products ranged between 100bp to 2200bp.

Carvalho *et al*. (2004) also examined genetic variability 79 landraces and two improved varieties of maize. Nine primers were selected as reliable amplifying ISSR markers. A total of 153 DNA fragments were scored with an average of 9.5 fragments per primer, 116 of which (75.8%) were polymorphic. Likewise, Amaral *et al.* (2011) also observed similar results in 52 accessions of *Z. mays* L. using fifteen primers, 137 bands were generated, out of which 122 were polymorphic (89.05%) and 15 were monomorphic (10.95%). The number of polymorphic bands ranged from 4, for the primer (GA)6CC, to 11 bands for the primers (GA)8T, (GA)8YC and (CTC)5RC.

DNA banding profile of individual plant DNA samples from 20 genotypes of *Z. mays* L. after amplification with ISSR primers are depicted in Plate 15 to 19.

4.3.2.7 Genetic Relationship and Cluster Tree Analysis on ISSR data:

The data obtain using ISSR primers (Appendix-IV) were further used to construct similarity matrix of *Z. mays* L. genotypes using 'Simqual' sub-programme of software NTSYS-pc. Dandrogram was constructed using similarity matrix values as determined from RAPD and ISSR data for 20 *Z. mays* L. genotypes using unweighted pair group method with arithmetic average (UPGMA) sub-programme of NTSYS-pc software.

ISSR similarity matrices revealed the relationship among them (Table 4.13). The similarity indices between different maize genotypes ranged from 0.36 to 0.87 i.e. 36-87%. The average similarity across all the genotypes was found out to be 0. 61, showing that genotypes were highly polymorphic. Maximum similarity value of 0.87 was observed in C.Sathi and Navjot. The low range of similarity showed that genotypes are genetically more diverse and highly polymorphic as also observed in banding pattern through isozymes as well as in D^2 analysis with five diverse clusters of genotypes.

4.3.2.8 Cluster tree analysis:

The ISSR cluster analysis of 20 *Z. mays* L. genotypes showed that they were mainly divided into two major clusters at a similarity coefficient of 0.47 (Fig. 4.9). Genotype Arawali was out-grouped from all other genotypes at a similarity coefficient of 0.47 and formed the first cluster. At a coefficient of 0.52 was the second cluster having all other genotypes. Genotype EC-3161 another solitary cluster at a similarity coefficient of 0.52. The second subcluster of cluster II was again divided into two main clusters at a similarity coefficient of 0.58 and out grouped genotypes HM-8, PM-4, from all other genotypes. The subcluster of cluster II was further divided into two clusters at a coefficient of 0.62. Cluster first contained only four genotypes *viz.*, HQPM-5, B.Sathi, K.Malan and PM-6 and second cluster was further separated into two groups- major and minor, at a coefficient of 0.64. Minor group consisted of EQH-16 and PM-5. While major group comprised the remaining 10 genotypes.

4.3.2.9 Principal Component Analysis (PCA):

ISSR data based on two and three dimension principal component analysis (Fig. 4.10 and Fig. 4.11 respectively) showed that most of the genotypes were separated into four clusters. The cluster I included five genotypes *viz.*, HQPM-1, EQH-63, Navjot and

C.Sathi while cluster II contained two genotypes PM-3 and Bio-9637. Cluster III contained genotypes PHEM-2 and PHM-2 and cluster IV included K.Malan and B.Sathi.

4.3.2.10 Genetic Relationship and Cluster Tree Analysis on Combined RAPD and ISSR data:

Perusal of the combined RAPD and ISSR similarity matrix data (Table 4.14) revealed that similarity indices for different genotypes ranged from 0.38 to 0.76 i.e. 38-76%. The average similarity across all the genotypes was found out to be 0.57, indicating average level of genetic similarity among the genotypes. The genotypes exhibiting the highest similarity included PHM-1 and EQH-63 having a similarity value of 0.76. However, Arawali and HQPM-5 were found to be genetically diverse with minimum similarity value of 0.36. These genotypes showed diversity in leaf number and plant height with morphological traits as well as banding intensity at isozymic level and were in different clusters as per D^2 analysis.

The number of loci and their genome coverage are critical in obtaining reliable estimates of genetic relationships among cultivars (Loarce *et al*., 1996). Also, highly effective mixed marker arrays (e.g., RAPD and ISSR) for genetic analysis require that they are informative and concordant with each other. When compared to other arbitrary primers like RAPDs, ISSR offer enormous potential for resolving intra-and inter-genomic relationship (Ziettkiewicz *et al*., 1994)

4.3.2.11 RAPD and ISSR markers based cluster tree analysis

The cluster analysis based on combined RAPD and ISSR data of 20 maize genotypes showed that they were mainly divided into two major clusters at a similarity coefficient of 0.48 (Fig 4.12). Genotypes HQPM-5 and EQH-16 out-grouped from all other genotypes at a similarity coefficient of 0.45 and formed the first cluster. At a coefficient of 0.52 was the second cluster having all remaining genotypes. Genotype Arawali was again out-grouped and formed another solitary cluster at a similarity coefficient of 0.52. The second subcluster of cluster II was further divided into two main clusters at a similarity coefficient of 0.54 and out grouped genotype EC-3161, from all other genotypes. The subcluster of cluster II was further divided into a major cluster at a coefficient of 0.58. Here, first cluster contained two genotypes C.Sathi and PM-3, and

second cluster being the major cluster and separated into two minor groups at a coefficient at 0.63. Minor I group contained 9 genotypes *viz.*, HQPM-1, HQPM-7, Navjot, EQH-63, PHM-1, PHEM-2, PHM-2, Bio-9637 and PM-5. While group II had 5 genotypes *viz.*, HM-8, PM-4, B.Sathi, PM-6 and K.Malan.

4.3.2.12 Principal Component Analysis (PCA):

PCA based on combined RAPD and ISSR data (Fig. 4.13 and 4.14 respectively) showed similar clustering of 20 genotypes of *Z. mays* L. as evident from the dendrogram. Most of the genotypes accumulated in single cluster while remaining were scattered throughout the plot. The major clusters contained 9 genotypes *viz,* Bio-9637, Navjot, HQPM-1, PHM-1, PM-5, HQPM-7, PHEM-2, PHM-1 and EQH-63.

4.4 Development of Sequence Characterize Amplified Regions (SCARs) Marker

SCAR markers could be a solution for the problem of reproducibility (Paran and Michelnore, 1993). In many cases, SCAR markers have been developed by converting dominant RAPD markers. SCARs can be developed by cloning and sequencing of polymorphic RAPD fragment, and then SCAR primers can be designed based on the insert sequence containing the RAPD primer. Conversion from RAPDs to SCARs enhances reliability and efficiency.

SCAR markers can also be used as a physical landmark in the genome (Paran and Michelnore, 1993). SCAR markers are co-dominant, mono locus and PCR-based markers that require the use of two specific primers. SCAR markers have many advantages including their specificity, low cost, ease and fast use. SCAR markers have been employed with success in plant and animal species identification (Yau *et al.*, 2002 and Bautista *et al.*, 2003). Usually SCAR markers are being developed from RAPD fingerprints (Arnedo-Andres, 2002 and Bautista *et al.*, 2003). Several researchers converted their dominant markers into co-dominant markers, such as SCAR marker from RAPD markers (Lahogue *et al.*, 1998 and Barret *et al.*, 1998). SCAR markers were developed for several crops including lettuce (Paran and Michelnore, 1993), rice (Naqvi

and Chattoo, 1996), brassica (Barret *et al.*, 1998a), wheat (Hernandez *et al.*, 1999), sugarcane (LuXiang *et al*., 2009) and maize (Shi *et al*., 2011)

4.4.1 Identification of Unique Band

In the present study, the primer OPP-05 gave bright, constant and unique band of 1.2 kb in genotype HQPM-5. This unique DNA band was excised from 1.2 percent agrose gel and DNA was purified by gel elution kit (Banglore Genei gel extraction kit) for further study. The purified 1.2kb fragment produced by OPP-5 random primer was cloned and sequenced by primer walking at Xeleris Pvt Ltd. Ahmadabad. From the sequence SCAR primer was designed using online software (Primer-3, www.simgene.com/primer3). The designed putatively genotype HQPM-5 specific SCAR primer pair could be gainfully utilized for confirmation/identification.

SCAR primer designed from OPP-5 primer amplified sequence.

Forward primer- 5'GT CCCCGGTAACGTGGTG3'

Reverse primer- 3'CCTGGAATAGATGGAAGAAGGTCC5'

Based on field and laboratory experiments *viz*., morphological, biochemical and molecular analysis it was concluded that 20 genotypes of *Zea mays* L. showed significant genetic diversity, since these belonged to variable genetic categories with diverse pedigrees from hybrids, composites and land races. Further, these have diverse geographical origins. Correlation and path analysis studies displayed that grain yield in maize appeared to be largely contributed by harvest index, biological yield per plant and grains per row. On the basis of *per se* performance HQPM-5, HQPM-1 and EQH63 turned out to be high yielding lines with superior grain protein content.

Grouping of genotypes based on morphological diversity and DNA fingerprinting did not follow similar classification. Noted differences in plant characters probably occurred over time due to the migration of alleles among genetic stocks, cross-pollination/or spontaneous mutation and/or free movement of plant materials from location to location. These variations might also be due to environmental fluctuations. Zymograms determined through isozymes *viz.*, esterase, peroxidase and superoxide dismutase classified all genotypes into two clusters.

The results further revealed utility of molecular markers in fingerprinting and depicting genetic diversity in *Z. mays* L. ISSR analysis produced more polymorphic bands than the RAPD analysis as also reported by Raina *et al*. (2001) and Shaw *et al*. (2009) in cultivars of *Arachis hypogea* and *Catharanthus roseus*, respectively. According to RAPD and ISSR similarity matrix data genetic diversity in *Z. mays* L genotypes ranged from 34-76% and 36-87%, respectively. This indicated that ISSR markers appeared more in determining the genetic diversity between genotypes as also noticed by Nagaoka and Ogihara (1977) and Aboel-Atta and Amhed (2009).

The unique bands in HQPM-5 as identified through RAPD was also further sequenced at Xeleris Pvt Ltd. Ahmadabad and developed SCAR primer for further genetic improvement.

Results clearly indicated that molecular analysis proved better as compared to isozyme analysis in fingerprinting of *Z. mays* L genotypes. Another important point emerged is that DNA markers could reveal very stubble genomic changes (mainly in repetitive non-coding DNA regions) which might be considered as intra-varietal variation on one hand, however, usually with no practical breeder's impact on the other hand (Wiesner *et al*., 2001).

The results of the present study could also be used as a stepping stone for evolving a well defined approach based on evaluation and characterization of genetic variation in *Z. mays* L. genotypes.

5. SUMMARY AND CONCLUSION

The present investigation entitled "Assessment of Morphological, Biochemical and Molecular Diversity in *Zea mays* L." was undertaken with 20 diverse maize genotypes comprising hybrids, composites and land races. The crop was raised during *kharif*, 2011 in randomized block design with three replications at Instructional Farm of Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India under following recommended agronomical and plant protection practices.

Observations were recorded on five randomly selected plants from each replication for eleven morphological/agronomical characters *viz.*, plant height, days to 50% flowering, days to maturity, cob length, cob girth, grain rows per cob, grain per row, hundred grain weight, biological yield, grain yield per plant, harvest index. Total soluble protein in grains were estimated by lowery's method (lowery *et al*., 1951).

Data so obtained were subjected to analysis of variance, genotypic variability parameters, correlation, path coefficient analysis and genetic divergence analysis. For biochemical characterization enzymes were extracted from young leaves (28 DAS) using standardized extraction buffer. Extracted enzymes were used to develop zymograms for three isozymes *viz.* esterase, peroxidase and superoxide dismutase.

Similarly for molecular analysis, DNA was isolated with CTAB extraction buffer method in seedling (21-25 DAS). DNA concentration and purity was checked by spectrophotometer and agarose gel electrophoresis. Isolated DNA was used as a template for DNA amplification using 16 RAPD and 10 ISSR primers as per standard techniques. The significant results based on field and lab studies are summarized as below:

- The analysis of variance revealed the presence of significant genetic variability among the genotypes for all eleven characters including grain protein content.
- On the basis of *per se* performance genotypes HQPM-5, HQPM-1 and EQH63 displayed superiority for grain yield, biological yield as well as for grain protein content.

- The magnitude of phenotypic coefficient of variation (PCV) was higher than their corresponding genotypic coefficient of variation (GCV) for all the characters. High GCV and PCV were recorded for test weight, grains per cob, grains per row and harvest index.
- High heritability (>80%) in broad sense was recorded for grain per cob, test weight, grain yield per plant and harvest index. Study also revealed high genetic advance as percentage of mean (genetic gain) along with high estimates of heritability and GCV for test weight, grain yield per plant, grains per cob, cob length, grains per row and harvest index.
- Association studies revealed a close agreement between genotypic and phenotypic correlations. Grain yield per plant was strongly correlated with days of 50% flowering, days of maturity, cob length, grains per cob, cob girth, grains per row and harvest index. Path coefficient analysis studies revealed that harvest index, biological yield and grains per row exhibited significant direct effect on seed yield in maize.
- On the basis of variability parameters, correlation and path analysis reveled that the selection for harvest index, grains per cob, cob length, cob girth and biological yield per plant, would enhance the productivity levels of *Z. mays* L.
- Using Hierarchical Euclidean cluster analysis, 20 maize genotypes were grouped into 6 divergent clusters, Cluster II was the largest contained 6 genotypes followed by cluster III which possessed 5 genotypes. The I, V and IV possessed 4, 3 and 2 genotypes, respectively.
- Intra-cluster distance between the genotypes was maximum for the cluster III followed in descending order by cluster I, II, IV and V. Inter – cluster distances ranged from 22.12 between I and IV to 91.55 between from I and III, indicating considerable amount of genetic variability. Maximum contribution to genetic divergence was through test weight, harvest index, grains per row and plant height.

- Conclusion based overall genetic variability revealed that biometrical estimates harvest index, biological yield and grains per row could enhance grain yield in maize.
- Mean values of different clusters for various characters revealed that HQPM-1, HQPM-5 and EQH-63 possessed high average values for leaf number, days to 50 percent flowering, days to maturity, cob length, cob girth, grain per cob, grain per row, grain yield per plant and harvest index. All these genotypes had high *per se* performance, therefore these could be utilized for future breeding programme.
- In the present study based on biochemical characterization using three isozymes *viz.*, esterase, peroxidase and superoxide dismutase exhibited presence of 8 diverse alleles in all. Only superoxide dismutase isozyme showed 63% polymorphism, which could be attributed to environmental condition. Cluster tree analysis was carried out by UPGMA method based on genetic distance. In all, genotypes were divided into two clusters at a similarity coefficient of 0.88. Isozyme similarity matrices of 20 genotypes of *Z. mays* L. ranged from 0.88 to 1.00. Genetic diversity and distance derived from isozyme analysis were low due to the small number of polymorphic alleles.
- The quantity of DNA isolated from various genotypes of *Z. mays* L. ranged from 1060 to 3685 µg/ml. genotype EQH-63 yielded the highest DNA (3685 µg/ml). Quality of isolated through CTAB method was found pure as indicated by the ratio of A260/A280 nm which ranged from 1.70 to 1.89.
- The Random Amplified Polymorphic DNA (RAPD) analysis showed high polymorphism (73.41). A total of 79 amplified fragments were formed by 16 primers out of which 58 were polymorphic bands. The total number of amplified bands varied between 2 (primer OPC-08, OPP-04, OPF-17 and OPP-03) and 9 (primer OPD-05) with an average of 4.9 bands per primer. The overall size of PCR amplified products ranged between 180 bp to 2500 bp.
- Jaccard's Similarity Coefficient Value for RAPD primers ranged from 0.34 to 0.76. Based on dandrogram generated through UPGMA method and principle

component analysis, most of the genotypes were classified into two main clusters. First cluster included 18 genotypes while second cluster possessed only two.

- The Inter Simple Sequence Repeat (ISSR) analysis showed high polymorphism (86.44). A total of 59 amplified fragments were formed by 10 primers out of which 51 were polymorphic bands. The total number of amplified bands varied between 2 (primer-808) and 8 (primer-852) with an average 5.9 per primer. The overall size of PCR amplified products ranged between 100 bp to 2200 bp.
- Jaccard's Similarity Coefficient Value for ISSR primers ranged from 0.36 to 0.87 with an average of 0.61. Based on dandrogram generated through UPGMA and principle component analysis, most of the genotypes mainly divided into two major clusters at a similarity coefficient of 0.47. Genotype Arawali was out-grouped from all other genotypes.
- Perusal of the combined RAPD and ISSR similarity matrix data revealed that the similarity indices for different genotypes ranged from 0.38 to 0.76 with an average 0.57, again indicating a very high level of genetic similarity among the genotypes. Based on dandrogram genotypes were mainly divided into two major clusters at a similarity coefficient of 0.48. Genotypes HQPM-5 and EQH-16 out-grouped.
- RAPD primer OPP-05 gave bright, constant and unique band of 1.2 kb in genotype HQPM-5 which was also further sequenced at Xeleris Pvt Ltd. Ahmadabad and developed SCAR primer for further genetic improvement.
- High genetic diversity among the maize genotypes appeared which might be on account of their variable categories with diverse pedigrees from hybrids, composites and land races. Further, these have diverse geographical origins. Further, correlation and path analysis studies displayed that grain yield in maize appeared to be largely contributed by biological yield and harvest index. On the basis of *per se* performance and all other parameters, HQPM-5, HQPM-1 and EQH63 turned out to be high yielding lines with superior grain protein content. Therefore, these could be gainfully utilized.

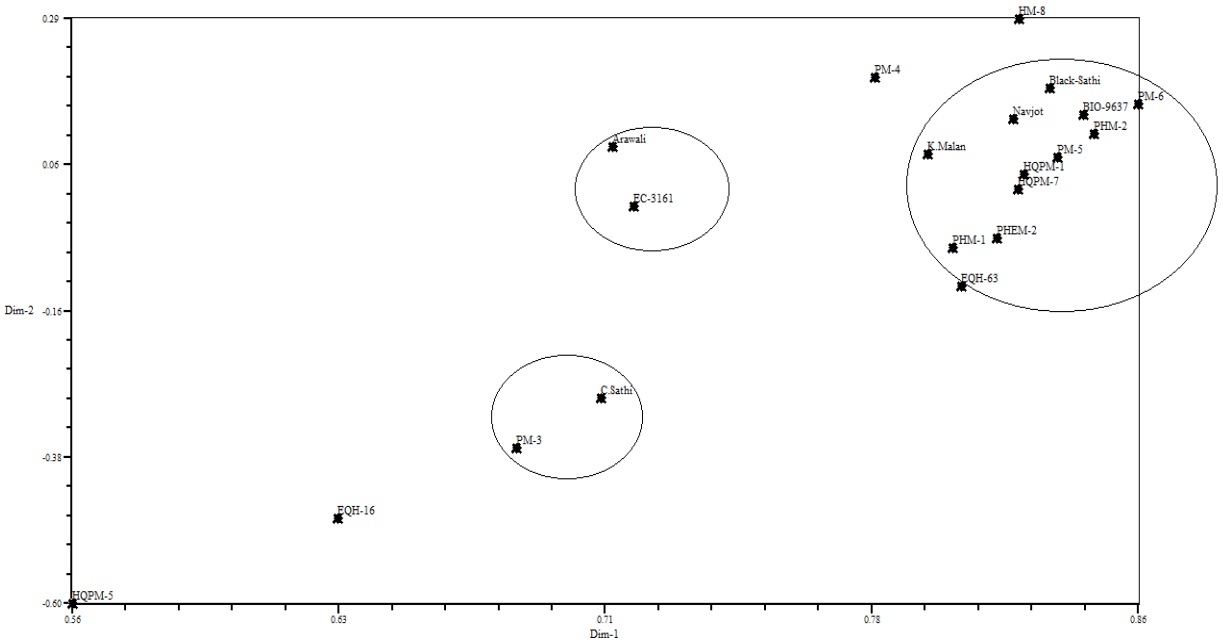
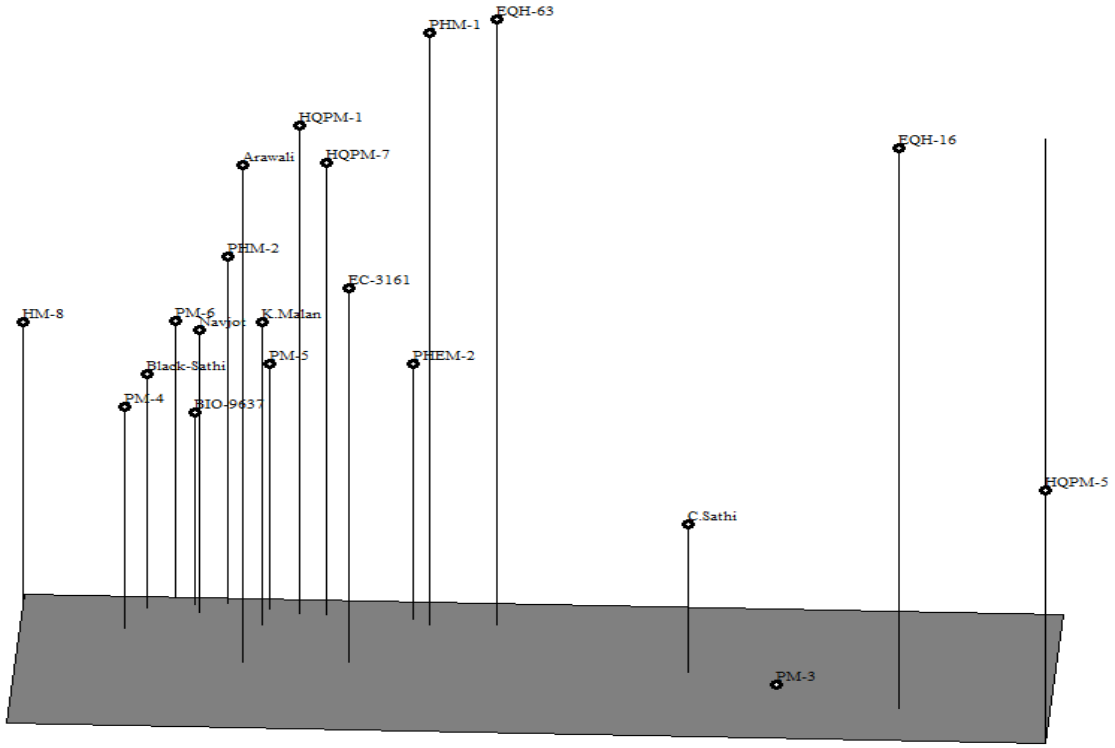
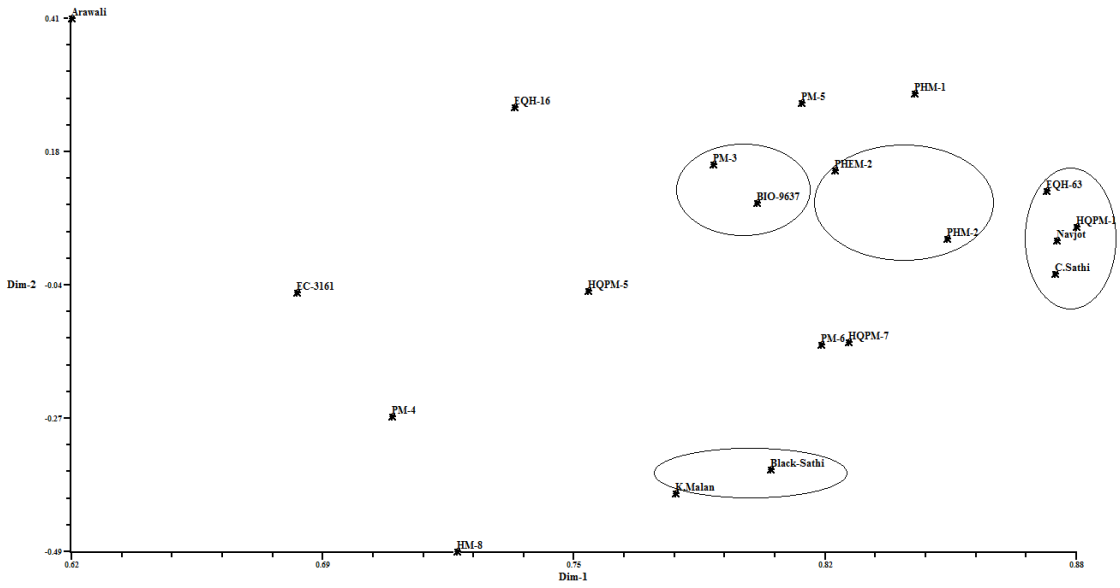


Figure 4.7: Two dimensional PCA (Principle Component Analysis) scaling of 20 genotypes of *Z. mays* L. using RAPD markers



➢ **Figure 4.8: Three dimensional PCA (Principle Component Analysis) scaling of 20 genotypes of *Z. mays* L. using RAPD markers**



➢ **Figure 4.10: Two dimensional PCA (Principle Component Analysis) scaling of 20 genotype of *Z. mays* L. using ISSR markers**

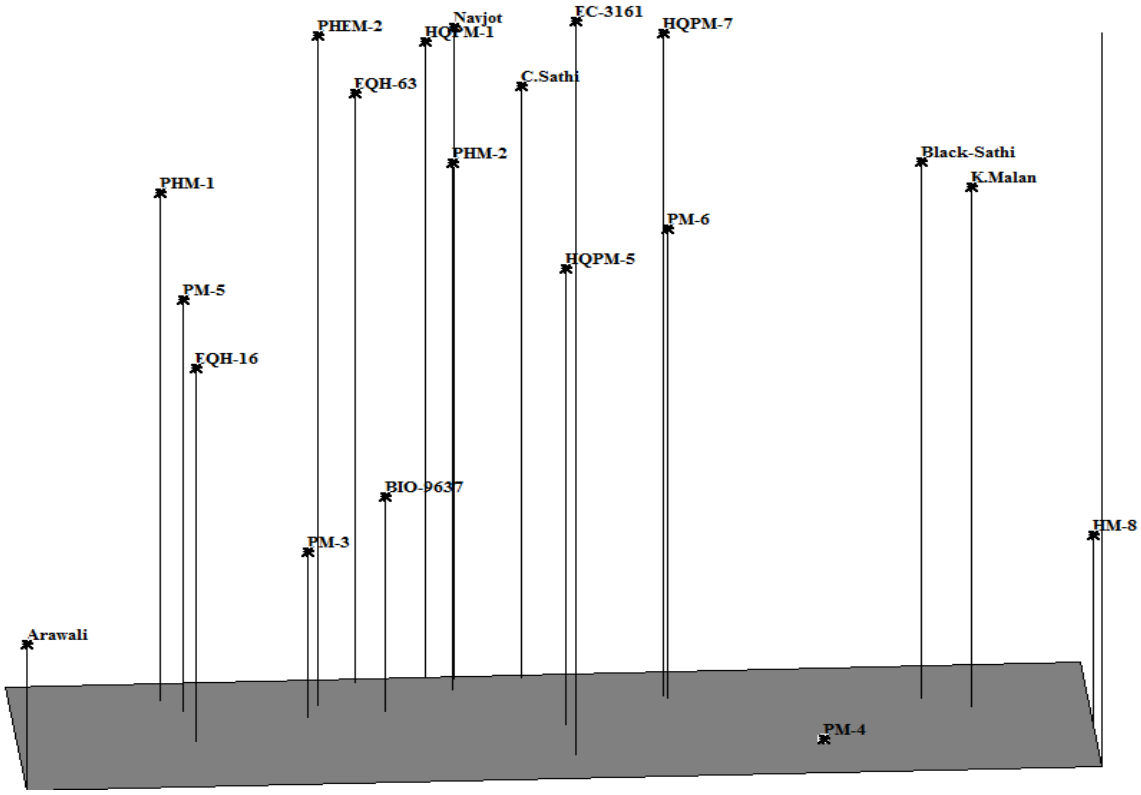


Figure 4.11: Three dimensional PCA (Principle Component Analysis) scaling of 20 genotype of *Z. mays* L. using ISSR markers

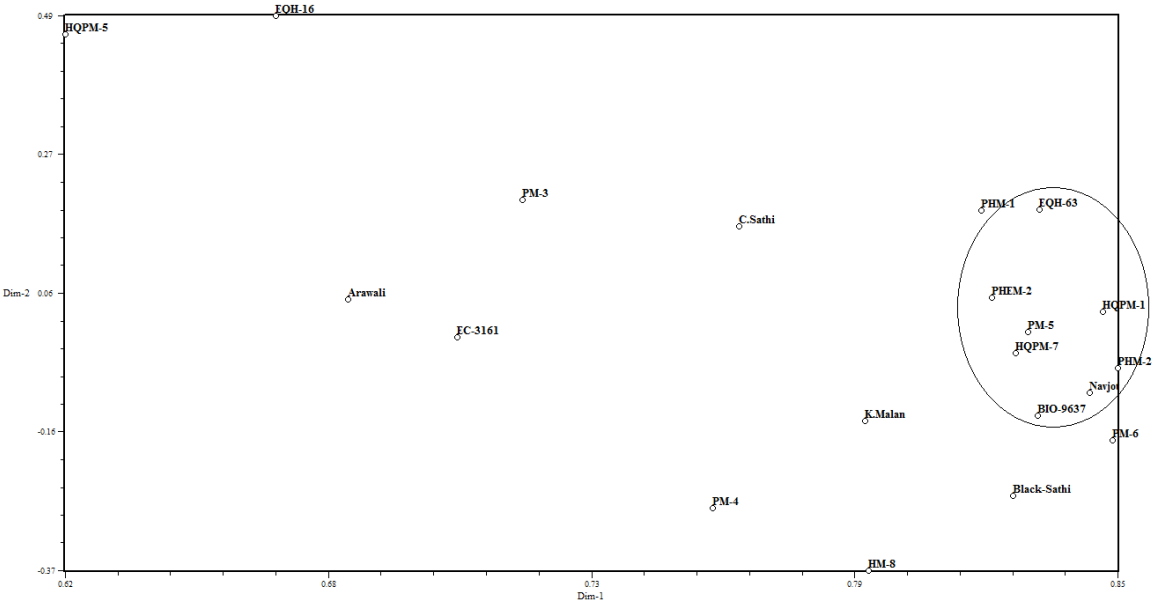


Figure 4.13: Two dimensional PCA (Principle Component Analysis) scaling of 20 genotype of *Z. mays* L. using RAPD and ISSR markers

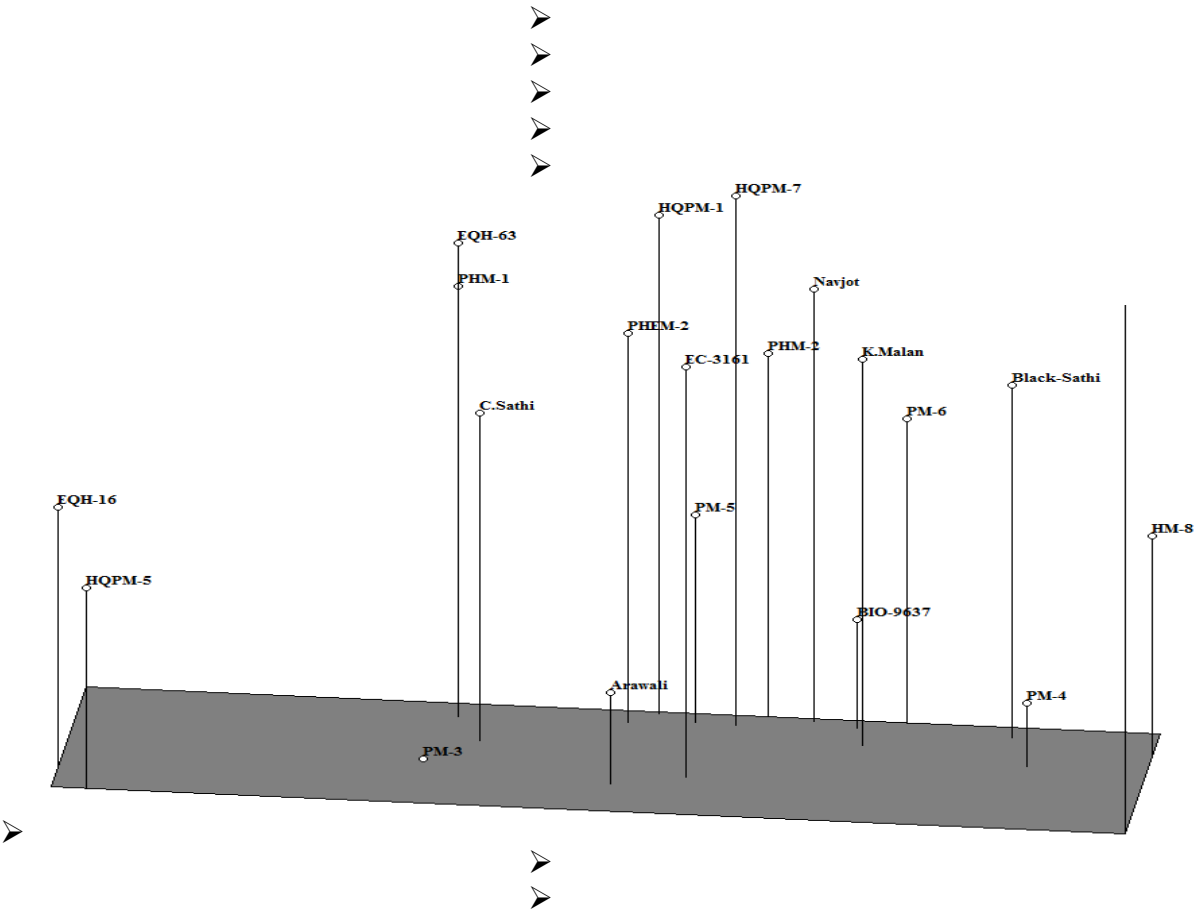


Figure 4.14: Three dimensional PCA (Principle Component Analysis) scaling of 20 genotype of *Z. mays* L. using RAPD and ISSR markers

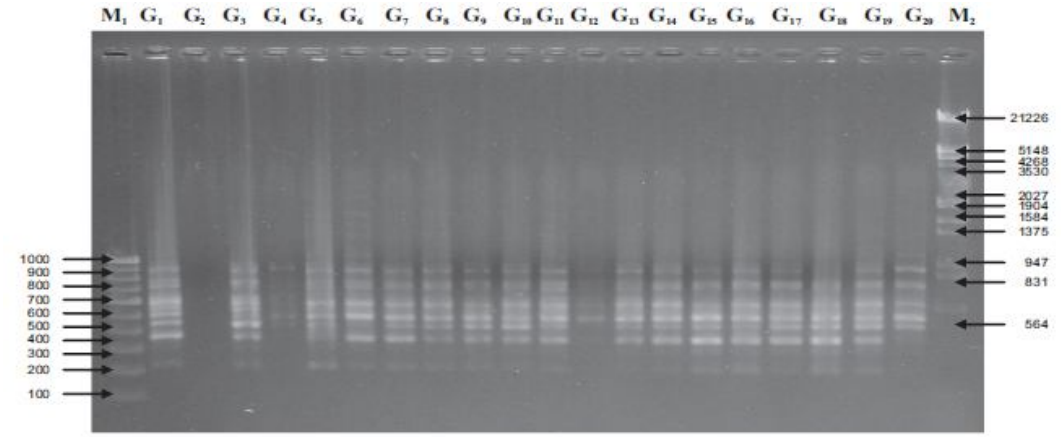


Plate-6: RAPD profile generated through OPP-01 (5' GTAGCACTCC 3')

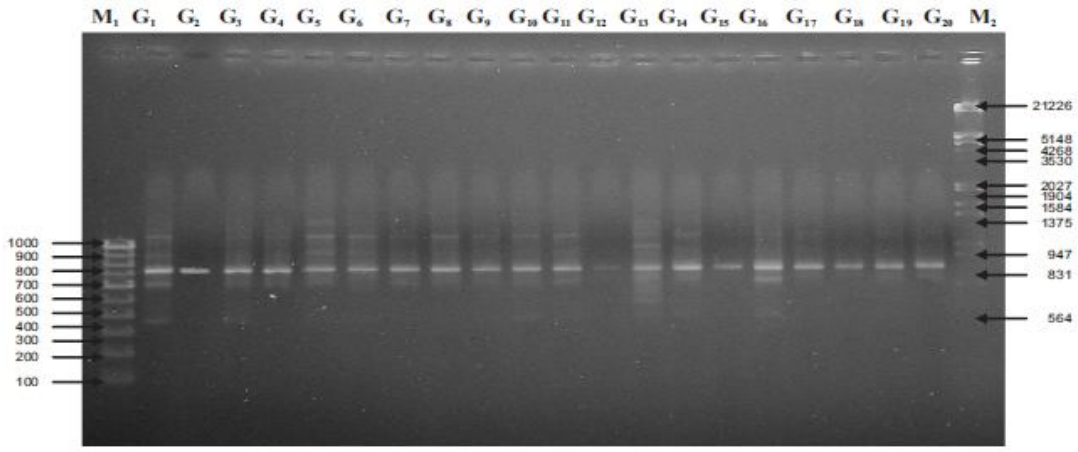


Plate-7: RAPD profile generated through OPA-01 (5' CAGGCCCTTC 3')

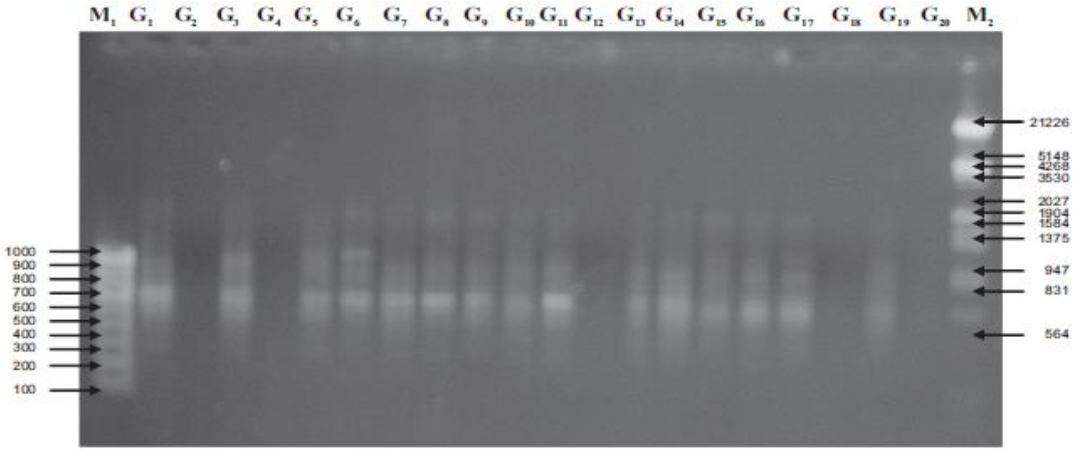


Plate-8: RAPD profile generated through OPP-04 (5' GTGTCTCAGG 3')

G_1-G_{20} represent following *Z. mays* L. genotypes

G_1- HQPM-1	G_6 - PHM-1	G_{11} - Arawali	G_{16} - Black Sathi
G_2 - HQPM-5	G_7 - PHEM-2	G_{12} - PM-3	G_{17} - PM-6
G_3- HQPM-7	G_8 - PHM-2	G_{13} - PM-4	G_{18} - Kumbhalgarh Malan
G_4- EQH-16	G_9 - BIO-9637	G_{14} - PM-5	G_{19}- EC-3161
G_5- EQH-63	G_{10} - HM-8	G_{15} - Navjot	G_{20}- Chanawada Sathi

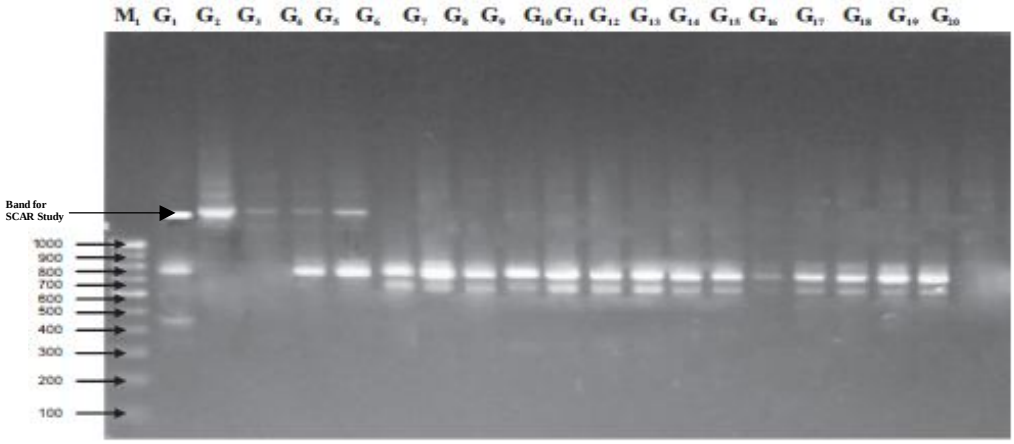


Plate-9: RAPD profile generated through OPP-05 (5' CCCCGGTAAC 3')

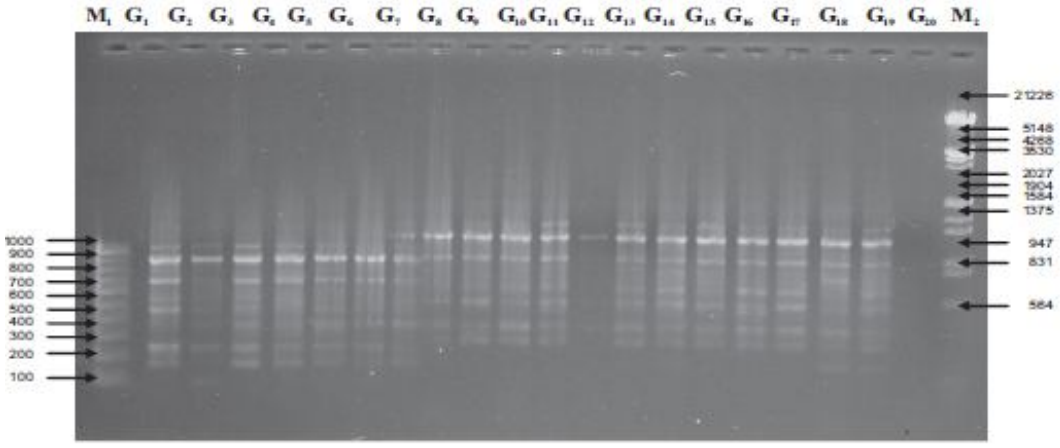


Plate-10: RAPD profile generated through OPD-05 (5' TAAGCGGACA 3')

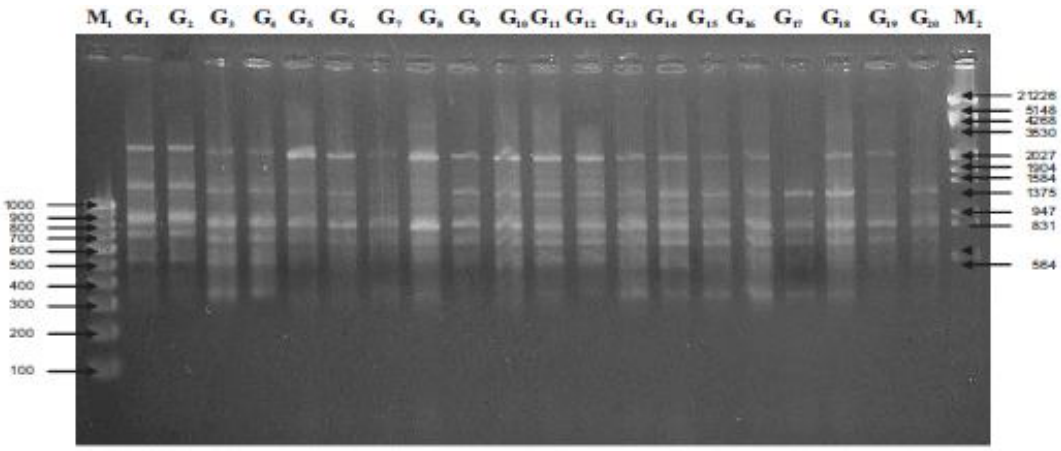


Plate-11: RAPD profile generated through OPD-07 (5' GTCCATGCCA 3')

G_1-G_{20} represent following *Z. mays* L. genotypes

G_1- HQPM-1	G_6 - PHM-1	G_{11} - Arawali	G_{16} - Black Sathi
G_2- HQPM-5	G_7 - PHEM-2	G_{12} - PM-3	G_{17} - PM-6
G_3- HQPM-7	G_8 - PHM-2	G_{13} - PM-4	G_{18} - Kumbhalgarh Malan
G_4- EQH-16	G_9 - BIO-9637	G_{14} - PM-5	G_{19}- EC-3161
G_5- EQH-63	G_{10} - HM-8	G_{15} - Navjot	G_{20}- Chanawada Sathi

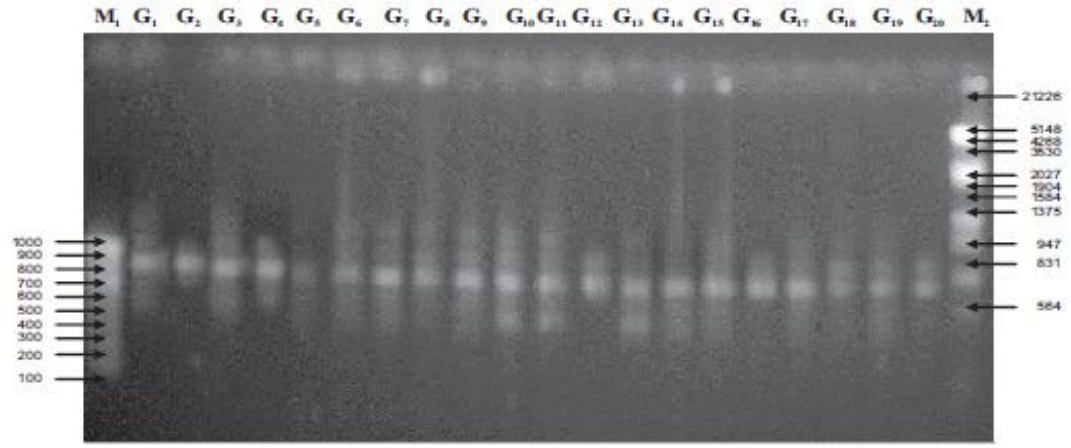


Plate-12: RAPD profile generated through OPP-02 (5' TCGGCACGCA 3')

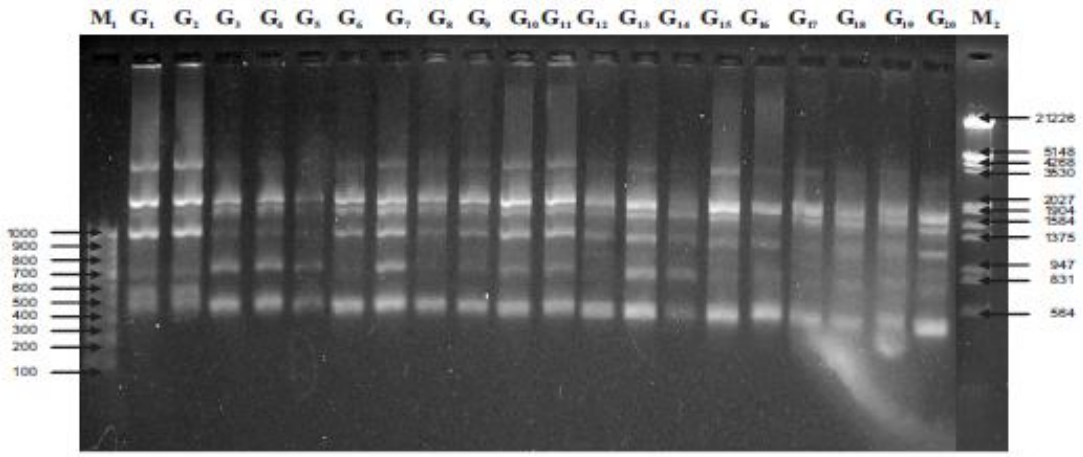


Plate-13: RAPD profile generated through OPP-12 (5' AAGGGCGAGT 3')

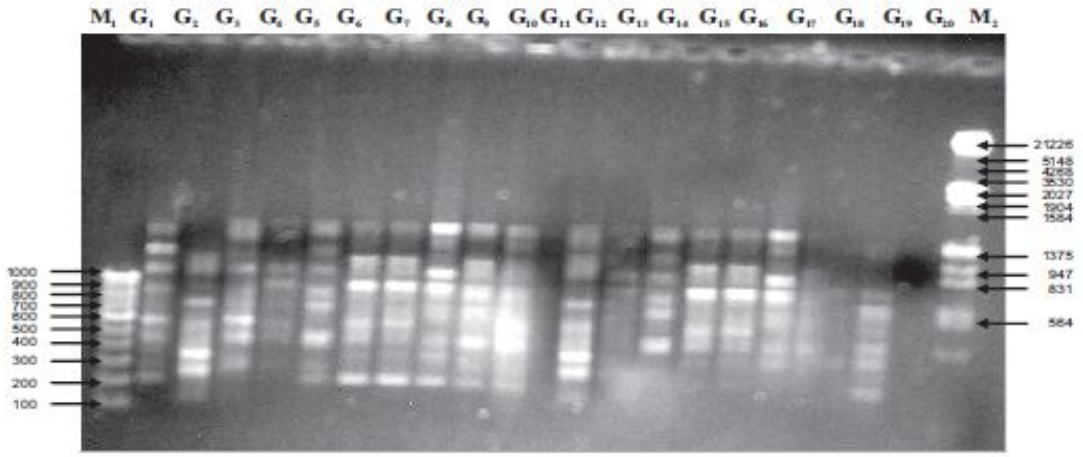


Plate-14: RAPD profile generated through OPP-10 (5' TCCCGCCTAC 3')

M_1 = 100 bp DNA Ladder M_2 = Lambda DNA/ *EcoR*I/*Hind*III double digest

G_1-G_{20} represent following *Z. mays* L. genotypes

G_1- HQPM-1	G_6 - PHM-1	G_{11} - Arawali	G_{16} - Black Sathi
G_2- HQPM-5	G_7 - PHEM-2	G_{12} - PM-3	G_{17} - PM-6
G_3- HQPM-7	G_8 - PHM-2	G_{13} - PM-4	G_{18} - Kumbhalgarh Malan
G_4- EQH-16	G_9 - BIO-9637	G_{14} - PM-5	G_{19}- EC-3161
G_5- EQH-63	G_{10} - HM-8	G_{15} - Navjot	G_{20}- Chanawada Sathi

Table 4.3: Genetic variability parameters for various characters in 20 genotypes of *Z. mays* L.

Characters	Mean and Standard error	Range	PCV %	GCV %	Heritability (broad sense) %	Genetic gain (5%)
Leaf number	13.03 ± 0.32	11.45-14.48	6.77	5.20	58.97	8.23
Plant height(cm)	193.36 ± 3.22	183.03-213.26	5.16	4.28	68.76	7.31
Days of 50% flowering	50.60 ± 1.49	42.33-59.33	9.85	8.42	73.13	14.84
Days of maturity	101.05 ± 2.23	80.66-112.66	8.39	7.47	79.26	13.71
Cob length (cm)	15.57 ± 0.60	11.06-18.53	15.07	13.45	79.74	24.75
Cob girth	12.29 ± 0.27	10.70-14.23	8.50	7.53	78.51	13.75
Grains/ cob	447.56 ± 29.21	291.33-653.66	25.53	22.89	80.39	42.28
Grains/row	32.36 ± 2.54	22.66-43.00	20.55	15.39	56.07	23.74
Test weight	20.14 ± 0.86	14.55-27.18	19.40	17.92	85.25	34.04
Grain yield /plant	80.03 ± 1.84	65.56-98.86	14.79	14.24	92.72	28.26
Biological yield/plant (g)	211.91 ± 4.52	201.33-223.80	4.19	1.98	22.47	1.94
Harvest index (%)	38.06 ± 1.23	30.94-47.00	15.74	14.70	87.24	28.30
Grain protein content	8.13 ± 0.32	6.12-10.70	6.17	5.87	54.93	8.11

Table 4.4: Genotypic (G) and phenotypic (P) correlation among different characters for grain yield in *Z. mays* L.

Characters	Correlation	Leaf number	Plant height	Days of 50% flowering	Days of maturity	Cob length	Cob girth	Grain/ cob	Grain/row	Test weight	Biological yield	Harvest index	Grain yield /plant
Leaf number	G	**1.00**	0.44**	0.12	0.054	0.07	-0.20*	0.45**	-0.13	-0.19	0.07	0.22**	0.18**
	P	**1.00**	0.32**	0.01	0.06	-0.00	-0.16	0.23**	-0.09	-0.16	0.02	0.14	0.11
Plant height	G		**1.00**	-0.48*	-0.36*	-0.17	-0.17	-0.17	-0.26*	0.37**	-0.05	-0.05	-0.12
	P		**1.00**	-0.37*	-0.32*	-0.11	-0.08	-0.14	-0.11	0.30	-0.16	0.00	-0.06
Days of 50% flowering	G			**1.00**	0.93**	0.46**	0.59**	0.82**	0.53**	-0.37*	-0.08	0.68**	0.67**
	P			**1.00**	0.74**	0.32**	0.46**	0.67**	0.37**	-0.31*	-0.06	0.54**	0.56**
Days of maturity	G				**1.00**	0.19**	0.42**	0.76**	0.25**	-0.47*	0.06	0.45**	0.41**
	P				**1.00**	0.15	0.28**	0.61**	0.16	-0.40*	0.10	0.33**	0.30**
Cob length	G					**1.00**	0.29**	0.45**	0.63**	0.17	-0.64*	0.74**	0.74**
	P					**1.00**	0.24**	0.36**	0.37**	0.14	-0.16	0.64**	0.68**
Cob girth	G						**1.00**	0.59**	0.51**	0.24**	-0.36*	0.66**	0.66**
	P						**1.00**	0.46**	0.36**	0.21**	-0.10	0.56**	0.59**
Grain/cob	G							**1.00**	0.45**	-0.31*	0.02	0.72**	0.74**
	P							**1.00**	0.53**	-0.25*	-0.02	0.60**	0.65**
Grain/row	G								**1.00**	-0.10	-0.16	0.54**	0.59**
	P								**1.00**	-0.07	-0.11	0.37**	0.45**
Test weight	G									**1.00**	-0.62*	0.10	0.04
	P									**1.00**	-0.31*	0.08	0.02
Biological yield	G										**1.00**	-0.57*	-0.49*
	P										**1.00**	-0.40*	-0.22*
Harvest index	G											**1.00**	0.99**
	P											**1.00**	0.95**

* Significant at 5 per cent and **Significant at 1 per cent probability level

Table 4.5: Path analysis at genotypic (G) and phenotypic (P) level showing direct and indirect effects (bold values) of various characters on seed yield in *Z. mays* L.

Characters	Path	Leaf number	Plant height	Days of 50% flowering	Days of maturity	Cob length	Cob girth	Grain\ cob	Grain/row	Test weight	Biological yield	Harvest index	Grain yield/plant
Leaf number	G	**-0.08**	-0.03	-0.01	-0.00	-0.00	0.01	-0.03	0.01	0.01	-0.00	-0.01	0.18**
	P	**-0.00**	-0.00	-0.00	-0.00	0.00	0.00	-0.00	0.00	0.00	-0.00	-0.00	0.11
Plant height	G	-0.05	**-0.11**	0.05	0.04	0.02	0.02	0.02	0.03	-0.04	0.00	0.00	-0.12
	P	-0.01	**-0.03**	0.01	0.01	0.00	0.00	0.00	0.00	-0.00	0.00	-0.00	-0.06
Days of 50% flowering	G	-0.03	0.12	**-0.24**	-0.23	-0.11	-0.14	-0.20	-0.13	0.09	0.02	-0.17	0.67**
	P	0.00	-0.02	**0.06**	0.05	0.02	0.03	0.04	0.02	-0.02	-0.00	0.03	0.56**
Days of maturity	G	-0.00	0.00	-0.01	**-0.01**	-0.00	-0.00	-0.01	-0.00	0.00	-0.00	-0.00	0.41**
	P	-0.01	0.06	-0.14	**-0.18**	-0.02	-0.05	-0.11	-0.03	0.07	-0.01	-0.06	0.30**
Cob length	G	-0.00	0.00	-0.00	-0.00	**-0.01**	-0.00	-0.00	-0.00	-0.00	0.00	-0.01	0.74**
	P	-0.00	-0.01	0.02	0.01	**0.09**	0.02	0.03	0.03	0.01	-0.01	0.06	0.68**
Cob girth	G	0.01	0.01	-0.04	-0.03	-0.02	**-0.07**	-0.04	-0.03	-0.01	0.02	-0.04	0.66**
	P	-0.01	-0.00	0.02	0.01	0.01	**0.06**	0.02	0.02	0.01	-0.00	0.03	0.59**
Grain/cob	G	0.05	-0.02	0.09	0.09	0.05	0.07	**0.12**	0.05	-0.03	0.00	0.08	0.74**
	P	0.02	-0.01	0.07	0.06	0.03	0.05	**0.11**	0.05	-0.02	-0.00	0.06	0.65**
Grain/row	G	-0.00	-0.01	0.02	0.01	0.03	0.02	0.02	**0.05**	-0.00	-0.00	0.02	0.59**
	P	-0.00	-0.00	0.01	0.00	0.01	0.01	0.01	**0.02**	-0.00	-0.00	0.01	0.45**
Test weight	G	-0.00	0.00	-0.00	-0.00	0.00	0.00	-0.00	-0.00	**0.00**	-0.00	0.00	-0.04
	P	0.00	-0.01	0.01	0.01	-0.00	-0.00	0.00	0.00	**-0.03**	0.01	-0.00	0.02
Biological yield	G	0.01	-0.00	-0.01	0.01	-0.10	-0.05	0.00	-0.02	-0.10	**0.16**	-0.09	-0.49**
	P	0.00	-0.02	-0.00	0.01	-0.02	-0.01	-0.00	-0.01	-0.05	**0.16**	-0.06	-0.22**
Harvest index	G	0.27	-0.06	0.83	0.55	0.90	0.81	0.88	0.66	0.13	-0.70	**1.21**	0.99**
	P	0.12	0.00	0.47	0.29	0.56	0.49	0.52	0.33	0.07	-0.35	**0.87**	0.95**

* Significant at 5 per cent and **Significant at 1 per cent probability level

Table 4.8: Cluster means and average (overall) for various characters in 20 genotypes in *Z. mays* L.

Cluster	Number of Genotypes	Leaf number	Plant height	Days to 50% flowering	Days to maturity	Cob length	Cob girth	Grain/ cob	Grain/row	*Test weight	Grain yield \plant	Biological yield	Harvest index
I	4	**13.65**	189.07	**56.58**	**109.08**	**17.55**	**13.04**	**605.58**	**36.83**	17.83	**96.52**	209.10	**46.32**
II	6	12.84	**195.70**	**52.55**	**104.88**	15.36	**12.78**	**474.00**	31.94	**20.37**	**81.55**	**213.86**	**38.81**
III	5	**13.08**	**194.58**	46.73	99.00	13.30	11.47	**403.20**	29.20	18.85	67.93	**214.71**	32.11
IV	2	12.29	184.43	50.00	99.83	**16.55**	11.61	313.33	**33.16**	18.25	69.91	**212.31**	33.02
V	3	**13.04**	**198.28**	45.55	86.88	**16.46**	12.13	347.44	32.00	**26.18**	**81.92**	206.79	**38.83**
Mean	4.00	13.04	193.36	50.60	101.05	15.57	12.29	447.56	32.36	20.14	80.03	211.91	38.06
TreatMSS		1.09	0.71	86.49	79.48	243.03	11.48	2.00	45005.49	33.17	36.64	512.32	43.11
ErrMSS		0.93	0.52	77.07	4.66	13.80	2.97	0.65	2376.43	30.81	7.69	32.39	36.90
F Ratio		1.16	1.35	1.12	17.04	17.61	3.86	3.08	18.93	1.07	4.76	15.81	1.16
Percent contribution towards variability		0.36	0.29	0.38	0.00	0.00	0.02	0.04	0.00	0.40	0.01	0.00	0.36

Table 4.11 : Jaccards similarity coefficient for RAPD profile generated by Agrose gel electrophoresis

Genotype	HQPM-1	HQPM-5	HQPM-7	EQH-16	EQH-63	PHM-1	PHEM-2	PHM-2	BIO-9637	HM-8	Arawali	PM-3	PM- 4	PM-5	Navjot	B. sathi	PM-6	K. Malan	EC-3161	C. Sathi
HQPM-1	1.00																			
HQPM-5	0.41	1.00																		
HQPM-7	0.72	0.42	1.00																	
EQH-16	0.49	0.49	0.51	1.00																
EQH-63	0.71	0.47	0.71	0.58	1.00															
PHM-1	0.74	0.40	0.67	0.54	0.75	1.00														
PHEM-2	0.67	0.43	0.65	0.46	0.64	0.65	1.00													
PHM-2	0.71	0.41	0.67	0.47	0.68	0.68	0.72	1.00												
BIO-9637	0.67	0.40	0.60	0.44	0.59	0.63	0.71	0.76	1.00											
HM-8	0.68	0.36	0.65	0.42	0.61	0.60	0.61	0.73	0.75	1.00										
Arawali	0.53	**0.34**	0.52	0.47	0.56	0.61	0.50	0.56	0.61	0.58	1.00									
PM-3	0.48	0.51	0.48	0.47	0.47	0.51	0.61	0.53	0.60	0.49	0.41	1.00								
PM- 4	0.59	0.36	0.65	0.43	0.59	0.54	0.55	0.61	0.68	0.73	0.57	0.52	1.00							
PM-5	0.67	0.41	0.64	0.49	0.63	0.62	0.68	0.66	0.74	0.62	0.60	0.57	0.72	1.00						
Navjot	0.68	0.36	0.72	0.44	0.61	0.64	0.67	0.69	0.66	0.63	0.53	0.52	0.63	0.70	1.00					
K.Sathi	0.65	0.41	0.68	0.44	0.60	0.60	0.65	0.66	0.67	0.73	0.54	0.52	0.69	0.69	0.71	1.00				
PM-6	0.65	0.42	0.66	0.47	0.65	0.64	0.65	0.72	0.73	0.76	0.61	0.53	0.65	0.71	0.71	**0.76**	1.00			
K. Malan	0.61	0.44	0.68	0.47	0.59	0.58	0.61	0.66	0.61	0.69	0.53	0.47	0.61	0.61	0.65	0.69	0.74	1.00		
EC-3161	0.53	0.40	0.53	0.44	0.55	0.55	0.61	0.57	0.58	0.59	0.54	0.42	0.48	0.57	0.54	0.61	0.60	0.53	1.00	
C. Sathi	0.54	0.43	0.57	0.48	0.54	0.53	0.58	0.53	0.55	0.47	0.42	0.61	0.49	0.55	0.58	0.54	0.57	0.56	0.49	1.00

Table 4.13: Jaccards similarity coefficient for ISSR profile generated by Agrose gel electrophoresis

Genotype	HQPM-1	HQPM-5	HQPM-7	EQH-16	EQH-63	PHM-1	PHEM-2	PHM-2	BIO-9637	HM-8	Arawali	PM-3	PM- 4	PM-5	Navjot	K.Sathi	PM-6	K. Malan	EC-3161	C. Sathi
HQPM-1	1.00																			
HQPM-5	0.68	1.00																		
HQPM-7	0.80	0.52	1.00																	
EQH-16	0.64	0.61	0.58	1.00																
EQH-63	0.80	0.66	0.72	0.69	1.00															
PHM-1	0.78	0.61	0.67	0.67	0.78	1.00														
PHEM-2	0.75	0.55	0.64	0.51	0.74	0.69	1.00													
PHM-2	0.75	0.65	0.67	0.58	0.78	0.69	0.75	1.00												
BIO-9637	0.69	0.56	0.54	0.52	0.62	0.66	0.66	0.73	1.00											
HM-8	0.56	0.53	0.60	0.45	0.54	0.47	0.48	0.59	0.60	1.00										
Arawali	0.48	0.46	0.39	0.56	0.52	0.59	0.45	0.45	0.54	**0.36**	1.00									
PM-3	0.62	0.51	0.57	0.53	0.61	0.68	0.65	0.69	0.75	0.55	0.56	1.00								
PM- 4	0.54	0.47	0.54	0.50	0.55	0.48	0.46	0.54	0.61	0.73	0.42	0.66	1.00							
PM-5	0.73	0.59	0.62	0.68	0.72	0.70	0.67	0.61	0.64	0.45	0.54	0.66	0.58	1.00						
Navjot	0.80	0.56	0.76	0.55	0.76	0.71	0.80	0.72	0.66	0.54	0.46	0.68	0.55	0.73	1.00					
B. sathi	0.68	0.57	0.70	0.47	0.64	0.59	0.65	0.62	0.57	0.68	0.40	0.56	0.59	0.54	0.71	1.00				
PM-6	0.67	0.62	0.62	0.55	0.69	0.67	0.58	0.61	0.64	0.63	0.51	0.57	0.51	0.62	0.70	0.70	1.00			
K. Malan	0.66	0.73	0.68	0.53	0.65	0.56	0.51	0.60	0.52	0.65	**0.36**	0.48	0.56	0.54	0.63	0.73	0.71	1.00		
EC-3161	0.55	0.48	0.56	0.47	0.57	0.52	0.62	0.59	0.48	0.50	0.40	0.41	**0.36**	0.50	0.59	0.54	0.56	0.51	1.00	

C. Sathi	0.75	0.58	0.76	0.57	0.74	0.72	0.71	0.72	0.69	0.56	0.48	0.71	0.54	0.67	**0.87**	0.75	0.73	0.66	0.58	1.00

Table 4.14: Jaccards similarity coefficient for RAPD and ISSR profile generated by Agrose gel electrophoresis

Genotype	HQPM-1	HQPM-5	HQPM-7	EQH-16	EQH-63	PHM-1	PHEM-2	PHM-2	BIO-9637	HM-8	Arawali	PM-3	PM- 4	PM-5	Navjot	K.Sathi	PM-6	K. Malan	EC-3161	C. Sathi
HQPM-1	1.00																			
HQPM-5	0.49	1.00																		
HQPM-7	0.75	0.45	1.00																	
EQH-16	0.53	0.53	0.53	1.00																
EQH-63	0.74	0.53	0.71	0.62	1.00															
PHM-1	0.75	0.46	0.67	0.58	**0.76**	1.00														
PHEM-2	0.69	0.47	0.65	0.48	0.67	0.66	1.00													
PHM-2	0.72	0.48	0.67	0.50	0.71	0.68	0.73	1.00												
BIO-9637	0.66	0.45	0.58	0.46	0.60	0.64	0.69	0.75	1.00											
HM-8	0.64	0.40	0.63	0.43	0.59	0.56	0.57	0.69	0.70	1.00										
Arawali	0.51	**0.38**	0.48	0.49	0.55	0.60	0.49	0.52	0.59	0.51	1.00									
PM-3	0.52	0.51	0.51	0.49	0.52	0.56	0.62	0.58	0.65	0.51	0.45	1.00								
PM- 4	0.57	0.40	0.62	0.45	0.58	0.52	0.52	0.59	0.66	0.73	0.53	0.56	1.00							
PM-5	0.69	0.46	0.64	0.55	0.65	0.64	0.68	0.64	0.71	0.57	0.58	0.60	0.68	1.00						
Navjot	0.71	0.43	0.73	0.47	0.65	0.66	0.71	0.70	0.66	0.60	0.51	0.57	0.60	0.71	1.00					
B. sathi	0.66	0.46	0.68	0.44	0.61	0.60	0.65	0.65	0.64	0.71	0.50	0.53	0.66	0.65	0.71	1.00				
PM-6	0.66	0.48	0.65	0.50	0.66	0.65	0.63	0.69	0.71	0.72	0.58	0.54	0.61	0.68	0.71	0.74	1.00			
K. Malan	0.63	0.52	0.68	0.49	0.61	0.57	0.58	0.65	0.59	0.68	0.48	0.47	0.60	0.59	0.64	0.70	0.73	1.00		
EC-3161	0.53	0.43	0.54	0.45	0.56	0.54	0.61	0.58	0.55	0.56	0.50	0.42	0.44	0.55	0.56	0.59	0.58	0.52	1.00	

C. Sathi	0.60	0.48	0.63	0.51	0.60	0.59	0.63	0.59	0.60	0.50	0.44	0.64	0.51	0.59	0.67	0.60	0.62	0.59	0.52	1.00

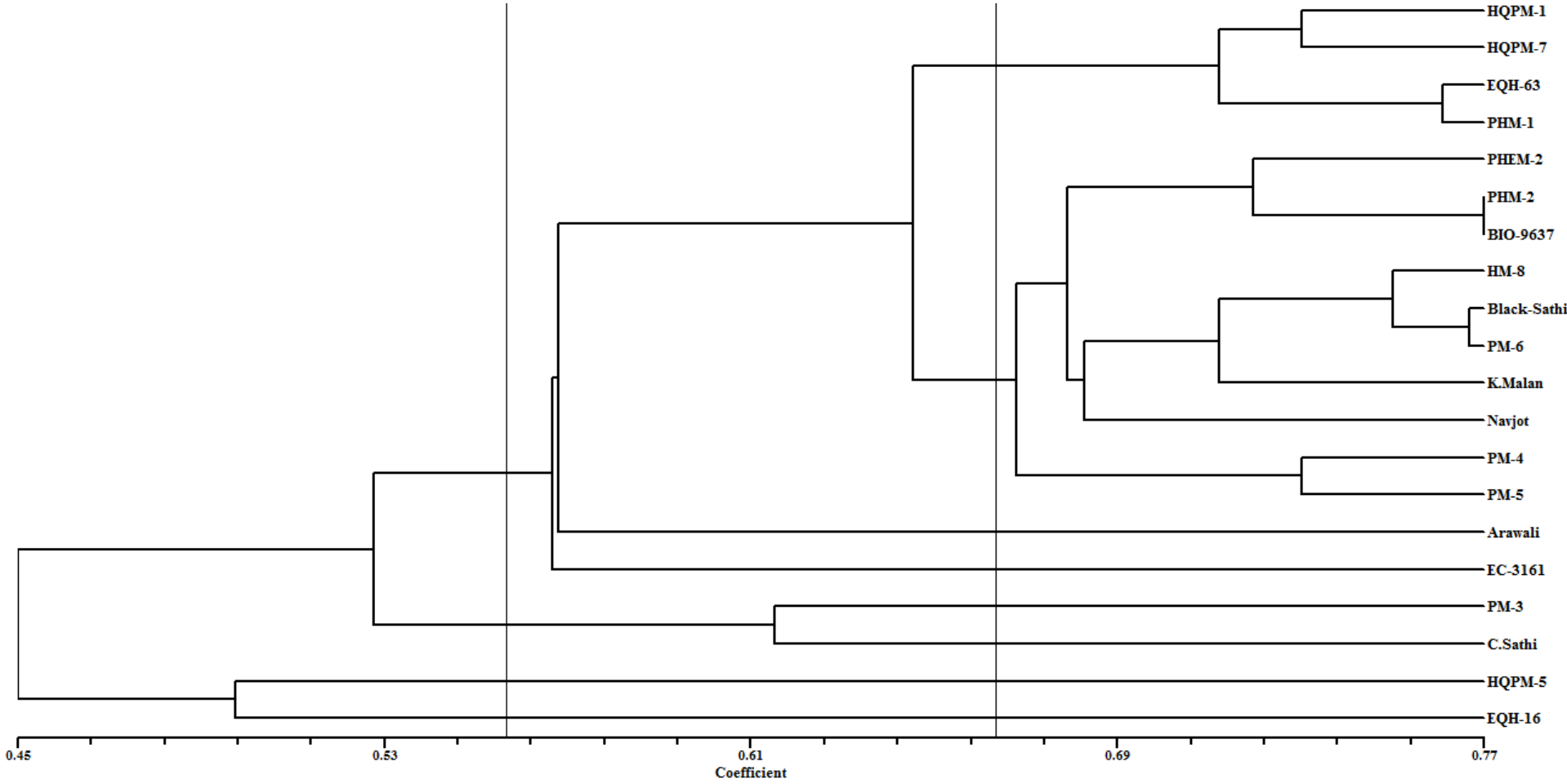


Figure 4.6: Dandrogram constructed with UPGMA clustering method of 20 genotypes of *Z. mays* L. using RAPD primers

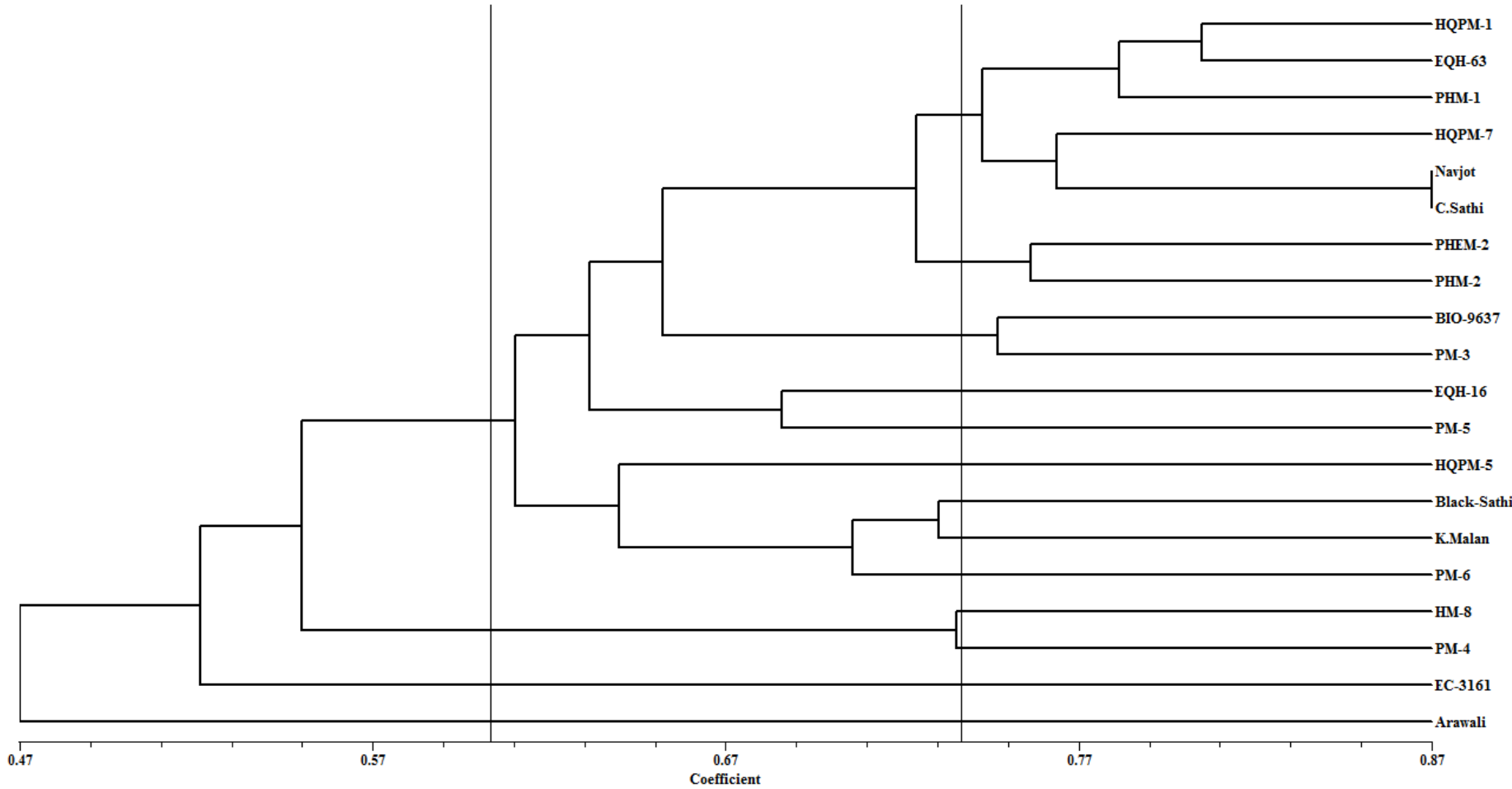

HQPM-1
EQH-63
PHM-1
HQPM-7
Navjot
C.Sathi
PHEM-2
PHM-2
BIO-9637
PM-3
EQH-16
PM-5
HQPM-5
Black-Sathi
K.Malan
PM-6
HM-8
PM-4
EC-3161
Arawali
0.47
0.57
0.67
0.77
0.87
Coefficient

Figure 4.9: Dandrogram constructed with UPGMA clustering method of 20 genotypes of *Z. mays* L. using ISSR primers

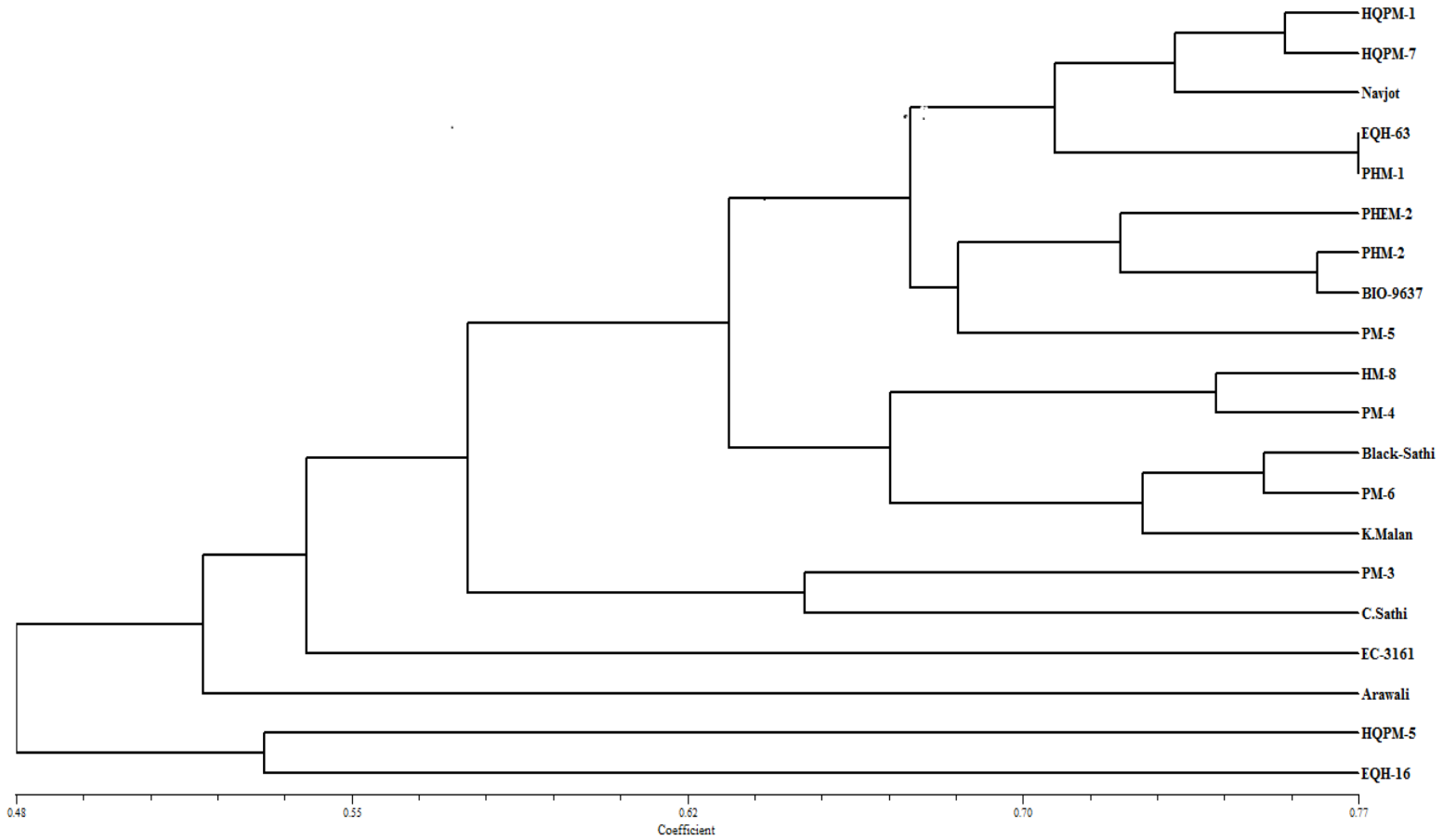


Figure 4.12: Dandrogram constructed with UPGMA clustering method of 20 genotypes of *Z. mays* L. using RAPD and ISSR primers

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