Journal of Applied Agricultural Economics and Policy Analysis, 2021, Vol. 4, No. 1, 1-17
Available online at http://pubs.sciepub.com/jaaepa/4/1/1
Published by Science and Education Publishing
DOI:10.12691/jaaepa-4-1-1

Identification of Genetic Distance of Exotic and Locally Developed Maize (*Zea mays* L) Inbred Lines

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Received December 09, 2020; Revised January 10, 2021; Accepted January 19, 2021

Abstract Development of hybrid varieties is one of the strategies to increase the productivity and production of Maize (*Zea mays* L.) in Sri Lanka. Heterosis is an important aspect to measure the hybrid vigor. Genetic distance is a main factor, which affects on heterosis. Identification of suitable parents using molecular markers gives many advantages over morphological evaluation. This study was conducted at the Molecular Biology Laboratory at Plant Genetic Resources Centre, Gannoruwa to identify distant parents using Simple Sequence Repeats (SSR) primers. Genomic DNA was isolated from selected 23 inbred lines including exotic and locally developed lines using modified CTAB method. Molecular characterization was done using four SSR primers; *umc1023*, *phi402893*, *phi 112* and *phi 041*. Amplified products were resolved in Polyacrylamide Gel Electrophoresis. Data were manually scored and analysis of genetic variation was carried out using the software POWEMARKER 3.1. Analysis revealed that polymorphism existed for all four SSR primers. Considerable genetic diversity was observed among the loci. Number of alleles per locus ranged from 2 to 3. Polymorphic information content (PIC) ranged from 0.24 *(phi112)* to 0.42 *(phi402893*). Gene diversity varied from 0.28 *(phi112)* to 0.46 *(phi402893).* Based on the values, it was revealed that there is a high genetic diversity present within the tested inbred lines. Genetic distance was calculated based on Nei's genetic distance and phylogenetic tree was developed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Tested 23 populations could be grouped into four clusters. Most of the exotic inbred lines grouped into one cluster revealed that there is less genetic distances present within the exotic lines. The genetic distance information obtained in this study will be useful to select better parents for development of maize hybrids with good performance.

Keywords: *genetic distance, maize hybrids, SSR markers*

Cite This Article: Jayasooriya J A U C, Dr. Wasala S K, and Zakeel M C M, "Identification of Genetic Distance of Exotic and Locally Developed Maize (*Zea mays* L) Inbred Lines." *Journal of Applied Agricultural Economics and Policy Analysis*, vol. 4, no. 1 (2021): 1-17. doi: 10.12691/jaaepa-4-1-1.

1. Introduction

Maize *(Zea mays* L; 2n=20) belonging to the family *Poaceae*. It is the second most important cereal crop in Sri Lanka. In world it ranks next to rice. It was first selected by American Indians during the period between 34th and 23rd century, with continuous improvements up to 15th century [1]. Colonial rules would have introduced the crop to Asia and its existence in Sri Lanka dates back to reign of ancient kings. Maize is an ingredient of over 500 different products and bi-products of foods, feed and industrial consumption groups. A being C4 plant, it is physiologically more efficient and has higher grain yield and wider adaptation.

In Sri Lanka it is mainly consumed in the form of boiled ears, dry grains boiled as rice or ground flour. According to the statistics, Sri Lanka requires around 200,000 mt. of dry grains annually of which about 80% is utilized in poultry feed production. However, less than 25% is being locally produced. Total extent of maize cultivation in year 2011 was recorded as 50,545 ha with the production of around 137,797 mt of grain in which about 65000-70000 farmer families are engaged [2]. Annual imports of maize in year 2011 was 8,244 mt. at a cost of Rs. 554.875 millions [2]. Local consumption is estimated as 33,000 tons and the requirements for *Thriposha* and other industries about 8,000 tons and 5,000 tons, respectively. In addition to that maize products such as corn flakes, corn oil, corn starch and corn flour annually imports for different uses.

With the increasing demand and the challenges in the crop cultivation, it is important to develop varieties with better performance. Plant Breeding has to play a big role in this. Therefore commercial breeding has shifted from open pollinated cultivars to hybrids. Hybrid is a progeny that result from cross between two parents [3]. Shull (1909) was first to report increase yields from F_1 generation between inbred lines. The better hybrid

combinations of selected lines give various advantages over other varieties such as higher yield, strength of stalk and roots, resistance to specific diseases. Several kinds of hybrids are possible, depending upon the number and arrangement of the parental inbred lines.

In 1950's the Department of agriculture attempt to develop maize hybrids [4]. But there was no demand for high cost seed for the subsistence farmers who preserved their own seed requirement after each crop [5]. But In the recent years, exotic hybrid varieties have become popular among farmers due to their high yield potential, uniform growth [3]. At present in Sri Lanka there is only one recommended locally developed hybrid variety,'*Samapath*. It had yield potential of 9 t/ha under current management conditions [6]. However, this yield performance is comparatively lower than the imported hybrid varieties.

Therefore producing of high yield hybrid varieties are one of the important strategies in maize variety improvement in department of Agriculture. Choosing of promising parental inbred lines are important for the production of successful hybrid. The value of any inbred line in hybrid breeding ultimately depends on its ability to combine with other inbred lines. This concept of combining ability has been very useful in hybrid breeding programs [7].

Heterotic effect of a hybrid can be defined as superiority of hybrids over its parents. This based on the genetic distance between the two parents and the level of adaptation of the parents. Heterosis is important to breeders because it is assumed that the cross of diverse parents will enhance the breeding success rather than similar parents [7]. Genetic distance estimates mostly by morphological markers.

Morphological markers are not an ideal for analyze genetic distance and diversity due to limited abundance, low degree of polymorphism, influenced by environments, and low throughput. Desirable molecular markers must abide with these properties, such as highly abundant and evenly distributed throughout the genome, highly polymorphic, neutral and not influenced by the environment [8].

A large number of molecular markers are now available; each has different advantages and drawbacks. Molecular markers that commonly used for diversity studies are RFLP (Restriction Fragment Length Polymorphism), and SSR (Simple Sequence Repeat). These marker types generally measure apparently neutral DNA variation, and are very useful in the analysis of genetic distance and phylogenetic relationships [8].

From these markers, simple sequence repeats (SSRs), or microsatellites, consist of tandemly repeated di-, tri-, or tetra- nucleotide motifs and are common feature of most eukaryotic genome. With their high level of allelic diversity, microsatellites are valuable as molecular markers, particularly for studies of closely related individuals [9]. This marker system have various advantages over other markers; such as multi-allelic nature, highly reproducibility based on PCR amplification technique, co-dominant nature, relative abundance extensive genome coverage small amount of starting DNA and no clones.

Selection of parents for hybrid variety development based on genetic distances can be utilized to develop productive hybrid varieties in Sri Lanka. Considering the highlighted advantages, this study was carried out to assess genetic distance of exotic and locally developed maize inbred lines using SSR markers.

2. Literature Review

2.1. Background

2.1.1. Taxonomy and Classification

Maize (*Zea mays L)* (2n=20) is a member of the kingdom plantae, division *Anthophyta*, class *Liliopsida*, order *Cyperales*, family *Poaceae* and genus *Zea* [10].

2.1.2. Origin, Domestication and Dissemination of Maize

The origin of maize is lost in antiquity. The plant is very highly specialized and could not reproduce itself without the aid of man. Wild maize has never been found by modern man. So no one knows when this plant originated but time of origin may be thousands of years ago [11].

Archeological and geological excavation and measures of radioactive decay of old ears of maize found in caves indicate that this plant must have originated at least 4,500 years ago in highlands of Mexico and America [12].

Maize domesticated from the wild grass *Teosintle* in Central America, spread northwards and southwards and was particularly abundant in the Aztec and Inca empires in Central America at the time when the New World was discovered [13]. In 1492, Columbus identified that maize was one of the good quality food consumed by Cuban people and later on he introduced maize in to Europe. Due to its high adaptability and productivity, maize spread rapidly around the world after the Europeans exported it [14].

Considering the extent of cultivation, maize first disseminated in United State of America, then Europe and later Asia and Africa. Colonial rulers would have introduced the crop to Asia and its existence in Sri Lanka dates back to period of ancient kings [6].

Table 1. Major Nutrient Composition of Maize Dried Grain

Nutrient	Percentage
Carbohydrate	73.76 %
Protein	7.20 %
Fat	3.99%
Minerals	1.04%
Moisture	12.81%
Fiber	1.20%

(Source: DOA, 2012).

2.2. Status of Maize Cultivation

2.2.1. World Status

Maize is the second most important cereal crop in the world after rice. It is cultivated in a wider range of environment than wheat and rice because of its greater adaptability. Major Maize producing countries are China, USA, India, Brazil and Argentina (FAOSTAT, 2010). The

world largest maize producer in 2011 was USA with the production of 313.91 million metric tons. China and Brazil occupied the second and third places respectively [12].

2.2.2. Sri Lankan Status

Maize is cultivated in many districts in Sri Lanka, mainly under rain fed conditions. It is considered primarily a dry zone crop and is one of the main crops cultivated in the highlands. It is cultivated as a mono crop as well as a mixed crop in both settled highlands and in the shifting type of agriculture practiced in highlands and *chena*. As well it is a popular crop in *chena* cultivation. The main demand for maize, aside from its consumption by the farming family, is in the livestock sector, where it contributes to about 25% of the poultry feed manufactured locally. Consequently, strategies are implemented to increase the extent of maize cultivation, as well as to develop high yielding varieties to improve the levels of production. Maize is still cultivated in Sri Lanka at a low level of technology, with seeds of local or mixed varieties, and minimum or zero inputs, particularly of fertilizers [2].

From the total cultivated areas in year 2011, 50,545 hectares were cultivated during *maha* and 8,639 hectares during *yala* season. The average production in *yala* was 33,306 mt and 137,797 mt in *maha*. Average yield in 2011 *maha* and *yala* seasons were 2.73 and 3.86 mt/ha respectively [2] Anuradhapura is the most popular district for maize cultivation. Apart from that, Moneragala, Badulla and Ampara districts also cultivate maize in large scale [15].

Table 2. Imports and Exports of Maize in Sri Lanka in Year 2011

	Import		Export	
	Quantity (Mt.)	Value ('000Rs)	Quantity (Mt.)	Value ('000Rs)
Maize (Seed)	1,234	304,957	-	-
Maize (Other)	7,011	249,918	0.5	38

[2].

2.2.3. Climate and Soil Requirement

Deep, loamy and fertile soils rich in organic matter, are preferred for satisfactory growth. Well-drained soils with adequate moisture supply are required for uninterrupted growth of this crop. By establishing the crops with the on-set of the rainy season, crops can be harvested before the depletion of soil moisture [15].

2.2.4. Maize Variety Improvement in Sri Lanka

Open pollinated maize varieties are still popular and commonly used by Sri Lankan farmers [6]. Initially, maize breeding programs were concentrated on developing open pollinated varieties. Open pollinated varieties are not performing high production capacity like hybrid varieties, although they have three major characters which cannot expected from hybrids. They are; easy to produce, simple and low cost seed production process, and farmers can get seeds from their previous cultivation [3].

There are various methods to develop open pollinated varieties via population improvement. High productivity, demand and requirement, height of plant, resistance to pest and diseases, avoid lodging, good combining ability, uniform growth and low variability are important characters considered in open pollinated varieties. Department Of Agriculture recommended four open pollinated varieties, *Bhadra*, *Ruwan*, *Aruna* and *Muthu* introduced in 1977, 1990 and 1992 respectively [16]. However, later variety *Muthu* was withdrawn from the recommendation list. *Sampath* is the only locally developed hybrid variety in the recommendation list [3]. Imported hybrid varieties such as Pacific II, Pacific 628, and Pacific 984 are commonly utilized by local farmers along those OPVs.

Table 3. Characters of *Sampath* Hybrid

Character	***Sampath***
Growth Habit	Erect
Plant Height At Maturity	18 5 Cm
Ear Height	110 Cm
Number Of Leaves/Plant	15
No. Of Ears/Plant	1-2
Length Of Ear At Maturity	22 Cm
Number Seed Rows/Ear	12-14
Number Of Seeds In A Row	44
Seed Color	Orange
Days To Mature	105
Average Yield	5-6 Mt.

[16].

2.3. Diversity in Maize

Maize has been described as one of the most diverse plants in the world and this diversity occurs at both the phenotypic and molecular levels. There are about 65000 accessions of maize in major germplasm banks of the world, of which more than 90% are *Z. mays*. The International Center for Wheat and Maize Improvement (CIMMYT) has the world's largest maize collection which includes 25,000 entries. Most of the diversity in maize remains undescribed, poorly understood and underutilized in modern crop improvement programs largely, because of the difficulty of identifying useful genetic variants hidden in the background of low yielding local varieties and landraces [17].

2.3.1. Morphological Diversity in Maize

Kuleshov (1933) reported on the world's diversity of phenotypes of maize. He found that maize had an extraordinary diversity of morphological and biological properties. Maize is divided in to various groups based on seed characters. There are seven major groups.

Pod corn - Kernel and ear enclosed in husk
Pop corn - Soft starchy center covered with hard shell.
Flint corn - Kernel covered with hard shell
Dent corn - Dent in the top of each kernal
Flour corn - Soft starchy yellow kernal
Sweet corn - Kernel has creamy texture

2.3.2. Genetic Diversity in Maize

Genetic diversity is the variation of heritable characteristics present in a population of the same species. It serves an important role in evolution by allowing a species to adapt to a new environment and be resistant to pest and diseases [18].

For a species to adapt to an ever-changing ecosystem, a significant level of variation has to be present. Those individuals that possess favorable characteristics will go on to reproduce and pass on their characteristics to the offspring's. Domesticated species often have low levels of genetic diversity. This is caused by the artificial selection or preferential breeding of crops and animals for traits that humans find preferable. This can have positive short-term results, such as a richer harvest [19].

World collections of maize comprise about 12,000 accessions that are represented in 256 races, of which about 30 are in the process of extermination [5]. It is estimated that only around 2% of the maize germplasm is utilized in breeding programs and an important fraction is cultivated and conserved by small landholder farmers. Most of the genetic variability is represented within and between landraces maintained by the traditional family farming systems [20].

2.3.3. Importance of Genetic Diversity

Recognizing and managing the genetic diversity is valuable because it is important to the species ability to adapt to changing environmental conditions over time, it is not the entire species that adapts in concert, but particular populations over time. Some populations may become better adapted than others; some may become extinct.

Also it is important to the species ability to colonize new areas and occupy new ecological niches. The upper elevation populations of plant species are often genetically differentiated from the lower-elevation populations. In these cases, genetic diversity allows the species to exist in substantially differing environments.

Genetic diversity is also correlated with some measures of fitness. Although the cause-effect connections are not all understood at present, there is substantial evidence that levels of genetic diversity are positively related to a species' ability to produce substantial and robust progeny and persist in the long term [21].

Genetic diversity is influenced by selection, mutation, migration, population size, and genetic drift and understanding how each of these factors influences the genetic diversity of a population is critical to the conservation of species [22].

Plant breeders have traditionally drawn on intra-species diversity to improve crop yields, protect cultivars from disease and pests, with the aim of increase productivity [23].

2.3.4. Utilization of Genetic Diversity for Crop Improvement

Understanding the molecular basis of the essential biological phenomena in plants is crucial for the effective conservation, management, and efficient utilization of plant genetic resources (PGR) [24].

The improvement of crop genetic resources depends on continuous infusions of wild relatives, traditional varieties and the use of modern breeding techniques. These processes all require an assessment of diversity at some level, to select resistant, highly productive varieties [24].

The frequent use of genetically similar maize breeding lines may narrow the genetic base of modern cultivars. Thus, a diverse collection of parent germplasm in breeding programs is very essential in crop improvement [25]. The knowledge of genetic divergence and its nature and degree serves a useful purpose to identify desirable parents for breeding program. Heterosis is considered to be an outcome of genetic complementation between divergent parents [26].

2.4. Hybrid Variety Development

Hybrid is a progeny that results from a cross between two genetically dissimilar inbred parents. It combines good characters from both parents that will segregate in the progeny, discouraging seed propagation by growers. The first crop from hybrid seeds will produce well, but it is not advisable to use second-generation seed for planting [26].

Hybrid varieties are developed to exploit the heterosis or hybrid vigor. Hybrid varieties are vigorous and highly productive because hybrid vigor is fully exploited in such varieties. All the individuals or plants of hybrid variety are genetically similar. Thus hybrid varieties are heterozygous but homogenous populations. They are highly uniform and more attractive because of homogenous nature. Hybrids have wider adaptability to environmental changes than inbred and pure line varieties due to high inheriting buffering capacity. And those can be developed in both cross and self pollinated species depending upon the magnitude of heterosis. However, hybrids are more common in cross pollinated species than self-pollinated crops. And hybrids are generally more tolerant to biotic and abiotic stresses than inbred or pureline varieties [16].

2.4.1. Heterosis

This is the phenomenon in which the cross of two stocks produces a hybrid that is superior in growth, size, yield or general vigor. Heterosis can be defined the superiority of F_1 hybrids in one or more characters over its parents. Hybrid vigor is used as synonym for heterosis. This term was first used by Shull in 1914 [27]. Heterosis is a major breeding tool for improving productivity potential of crop plants [26].

There are two main theories which have been advanced to explain the heterosis. Dominance hypothesis and over-dominance hypothesis. The epistasis is also considered to be associated with heterosis [27].

According to dominance hypothesis, heterosis is the result of superiority of dominant alleles when recessive alleles are deleterious. The deleterious recessive genes of one parent are hidden by the dominant gene of another parent and the hybrid exhibit heterosis. This is the most acceptable theory. Over dominance hypothesis explains heterosis is the result of superiority of heterozygote over its both homozygous parents and epistasis refers to the interaction between alleles of two or more different loci. It is also known as a non-allelic interaction [27].

Assuming heterosis as a function of heterozygosity, it can be considered as a function of parental diversity. Several workers have emphasized the importance of genetic divergence for the selection of desirable parents in many crops [26].

2.4.2. Types of hybrids

Single cross hybrids- The hybrid progeny from a cross between two inbred or varieties is referred to as single

cross hybrid. In cross pollinated species this hybrids are produced by crossing between two inbreds whereas in self pollinated species they are developed from a cross between two homozygous varieties [28].

Double cross hybrids - The hybrid progeny from a cross between two single crosses. These hybrids are commonly used in maize and sugar beet [28].

Three way cross hybrids- Hybrid progeny between a single cross and inbred lines. Single cross is used as female and inbred as male [28].

2.4.3. Development of Hybrids in Maize

Development of hybrids differs from species to species. The production of hybrids in maize consists of three steps,

1. Development of inbred by selfing of heterozygous population or by doubling of haploids. Various populations can be used for selfing. Superior plants based on vigor, disease resistance and yield are selected and by selfing desirable genes in homozygous condition can be fixed while eliminating genotypes with undesirable deleterious genes. Inbreds can be developed from haploids by doubling the chromosome number through colchicine treatment. The vigor which is lost during inbreeding is regained when two unrelated or diverse inbred lines are combined to develop *F1* hybrid [29].
2. Evaluation of inbred. The value of any inbred line in hybrid breeding ultimately depends on its ability to combine with other lines to produce superior hybrids. Very few productive lines prove to be good combine as well. Two methods are available:
   i. Top cross method- A cross between inbred lines and open pollinated variety. This method is used for measuring General Combining Ability
   ii. Single Cross method- This method is used to measure the specific combining ability of inbred which is selected on the basis of the performance of top cross performances. The best performing single crosses are identified for release a variety for a use in the production of double cross hybrids [29].
3. Production of hybrid seeds- After the identification of suitable parents to the crosses, the production of hybrid seed is taken on a commercial scale which involves the multiplication of genetically pure quality seeds of parental lines and their crossing to obtain hybrid seeds of acceptable genetic purity [29].

2.5. Limitation of Methods of Morphological Characterization

The current characterization of plant germplasm collections relies strongly on morphological descriptors. Morphological descriptors are easy to study, relatively low cost to evaluate and reliable. However, the use of morphological characterization give some limitations, such as (a) limited polymorphism, lowering the potential success of an extended classification approach, which would require a high number of descriptors in order to compensate the small number of morphotypes. (b) Environmental influence on the phenotype, making even more complex the process of evaluation and information exchange. Here one should be careful with false positive when the environment affects specific morphotypes [30]. As a result, phenotypic variations occurring due to change in the environmental conditions could be erroneously scored in the process of germplasm characterization. Morphological markers require the observation of the whole mature plant for the characterization of plants particular germplasm [9]. Therefore, plant breeding programs where the breeders have to await the necessary traits to appear for long duration of time is highly disadvantageous. (c) Impact of a morphological descriptor in the viability of the individual [30].

The epistasis interaction existing among morphological marker loci or between morphological marker loci and genetic background is another major limitation in the use of the morphological markers for germplasm characterization. Epistasis prevents distinguishing all the genotypes associated with the morphological marker and also reduces the number of morphological markers that could be used in scoring [9]. Pleiotropism and the late onset of some morphological markers during plant development also render unequivocal assessment difficult [22]. Thus, phenotypic markers are not ideal for phylogenetic studies due to the limited abundance, low degree of polymorphism, influenced by the environment (G×E), and low throughput [8]. As a result of that they are not utilized singly, but in conjugation with molecular markers.

2.6. Molecular Markers

A molecular marker is a short stretch of nucleotide that is inherent. Molecular markers help identify traits like maturity or height along the DNA trail. Using molecular markers, pioneer researchers can better predict which plants have beneficial traits. This saves time because the first selection is made in the lab even before field trials begin. As a result, breeders begin field trials with an improved pool of candidate hybrids those are more likely to succeed in customers' fields.

Those Molecular markers rely on a direct DNA assay, rather than, for example, on an inherited phenotypic trait (e.g., flower color in pea, *Pisum sativum*, or semi-dwarfness in cereals, referred to as "morphological markers") or a protein-based assay of a gene product (e.g., amylase or seed storage proteins - "biochemical markers") [31]. Desirable molecular markers should be highly abundant and evenly distributed throughout the genome, highly polymorphic or with high Polymorphic Information Content (PIC), high multiplexing ratio, co-dominant and neutral nature [8].

Variation among individuals in a population or among populations in a species derived from genes and or environmental effects can be easily evaluated using a variety of markers. The discovery of proteins and enzymes that encoded by genes, let the utilization of *isozymes* and other proteins as marker systems for genetic analysis. Although protein markers circumvent the effects of environment, they have the drawbacks of a limitation in the number of detectable isozymes as well as tissue and development stage specificity [22].

DNA marker systems, which were introduced in 1980s for genetic analysis, have many advantages over the traditional morphological and protein markers that are used in genetic and ecological analyses of plant populations: firstly, an unlimited number of DNA markers can be generated; secondly, DNA marker profiles are not

affected by the environment, and thirdly DNA markers, unlike *isozymes* markers, are not constrained by tissue or developmental stage specificity [22].

Southern blot based markers are the first generated DNA marker systems. Restriction Fragment Length Polymorphisms (RFLPs) result from point mutations in restriction enzyme recognition sites. The second generation DNA markers for genetic analysis were those derived from PCR (Polymerase Chain Reaction). The PCR revolutionized genetic and ecological analyses of populations in several ways because it had two major advantages over Southern blot based markers. Firstly, it requires only a small amount of DNA and secondly, it is inexpensive and simple enough that experiments can be carried out rapidly on a large scale. The many PCR-marker techniques that have been developed, RAPD, AFLP and SSR are the major systems, with the other systems being the modifications of these three [22].

2.6.1. Advantages of Molecular Markers over Morphological Markers

Molecular markers are neutral markers not being affected by environmental or growth conditions, whereas the expression of morphological traits is affected by variation in environmental conditions. Molecular markers are not affected by epistasis interaction although the morphological markers are affected by interaction among morphological marker loci or between morphological marker loci and genetic background hence, resulting in the prevention of distinguishing all genotypes associated with the morphological markers and limitation of the number of morphological markers that could not be suited.

Molecular markers can be obtained from any plant tissue at any given stage of the life cycle. Therefore, selection of plants can even be done at seedling stages of the plant of interest, avoiding the necessity to linger until the plant is fully mature, as in the case of morphological markers. Early selection allows breeding programmes to speed up resulting in the increase in the rate of genetic gain of a breeding programme [32].

Only a small amount of plant material is required for the detection of polymorphism among individuals within a species or population. PCR based techniques help to increase available amount of DNA exponentially. Unlike in the morphological markers, the power of discrimination is very high in molecular markers, allowing easy separation of closely related species or varieties [9].

2.6.2. Non PCR Based Molecular Markers

Restriction Fragment Length Polymorphism (RFLP)

RFLPs are bands that correspond to DNA fragments, within the range of 2-10 kb, DNA and fragments are separated by agarose gel electrophoresis and are detected by Southern blot hybridization using a labeled DNA probe and variation of DNA sequence affecting the absence or presence of recognition sites of restriction enzymes, and insertions and deletions within two adjacent restriction sites, form the basis of length polymorphisms. RFLPs can be applied in diversity and phylogenetic studies. RFLPs are widely used in gene mapping studies.

Maize breeding based on RFLP

The RFLP in plant genetics was recognized by plant breeders under marker assisted selection in long term breeding programs as quality control markers and as a tool for the characterization of different varieties. Optimal use of RFLP technology for these purposes involves the construction of detailed RFLP linkage maps for the crops of interest. A high frequency RFLP maps has been constructed for maize [33]. These markers were used to map both simple and complexly inherited traits, allowing the calculation of genetic distance based on genome analysis in maize [34]. This is useful for predicting forage yield of crosses between lines from the same germplasm group or crosses including line combination from the same as well as different heterotic group [35]. RFLP is used to characterize genetic diversity among different hybrids in maize. RFLP data revealed additional polymorphism, thereby exhibiting a greater capacity to discriminate between maize hybrids than was possible with biochemical data [22].

2.6.3. PCR Based Molecular Markers

Many advances in molecular marker technology have come through applications of PCR method. In PCR, a thermo-stable DNA polymerase enzyme makes copies of a target sequence beginning from two small pieces of synthetically produced DNA (primers) which are complimentary to sequences bracketing the target fragment. Though iterations of the process with heating to separate the double stranded DNA molecules and cooling to allow the primers to re-anneal, the target sequence is exponentially amplified. PCR based markers require less amount DNA per assay than RFLPs and more compatible with automated high-throughput genotyping (i.e. the ability to process large numbers of samples quickly and efficiently) [9].

Randomly Amplified Polymorphic DNA (RAPD)

RAPDs are DNA fragments amplified by the PCR using short synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1-10 genomic sites simultaneously. Polymorphism mainly occurs due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites.

RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers [22].

RAPD for maize breeding

In maize arbitrarily primed PCR is used to identify inbred parents of hybrid maize plants. Despite the lack of direct correlation, the RAPD could be a useful tool for the selection of parents for testing hybrid combinations. This technique has greater potential in allocating genotypes of unknown origin to known heterotic groups [9].

Amplified Fragment Length Polymorphism (AFLP)

AFLPs are molecular markers derived from the selective amplification of restriction fragments. This marker is expected to be highly polymorphic. As the first step, Genomic DNA is digested with a pair of restriction enzymes and oligonucleotide adaptors are ligated to the ends of each restriction fragment. The fragments are amplified using primers that anneal to the adopter sequence and extend into the restriction fragments. Only a portion of restriction fragments will be within the range of

sizes than can be amplified by PCR and visualized on polyacrylamide gels (between 50 and 350 bp). AFLPs have many of the disadvantages of RAPDs, such as high degree of technical skill etc., but have much better reproducibility. Using manual gels, AFLP bands are detectable using silver stain, or by labeling of the primer with radioactive isotope. Alternatively, for higher throughput, AFLPs can be detected with an automated DNA sequence by using fluorescently labeled primers [9].

Simple Sequence Repeats (SSR) /microsatellites

Microsatellites represent tandem repeats, but their repeat motifs are shorter (1-6 base pairs). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20-25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small insert genomic library, screening the library with a synthetically labeled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers that have already been designed for closely related species may be used. Chromosome slippage during DNA replication or slipped strand mis-pairing is considered to be the main causes of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis [36].

The advantages of microsatellites include the co-dominance of alleles, their high genomic abundance in eukaryotes and their random distribution throughout the genome, with preferential association in low-copy regions [36]. Because the technique is PCR-based, and only low quantities of template DNA (10-100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap [9]. This decreases significantly the analytical costs.

Main drawbacks of microsatellites are that high development costs are involved. Since it is species specific primers, if adequate primer sequences for the species of interest are unavailable, it is not specific to apply. Although microsatellites are in principle co-dominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an under estimation of heterozygosity. Furthermore, the underlying mutation model of heterozygotes may be confused with homozygotes. However, the interpretation may be clarified by including appropriate reference genotypes of known band sizes in the experiment.

In general microsatellites show a high level polymorphism. As a consequence, they are very informative markers that can be used for many population genetic studies, ranging from the individual level (e.g. clone and strain identification) to that of closely related species. Conversely, their high mutation rate makes them unsuitable for studies involving higher taxonomic levels. Microsatellites are also considered ideal markers in gene mapping studies [22].

2.6.4. Utilization of Molecular Markers for Diversity Studies

Analyses of genetic diversity are usually based on assessing the diversity of individuals. Genetic variability within a population can be assessing through,

1. The number (and percentage) of polymorphic genes in the population.
2. The number of alleles for each polymorphic gene.
3. The proportion of heterozygous loci per individual.

Molecular methods, such as DNA analysis, directly measure genetic variation giving a clear indication of the levels of genetic variation present in a species or population without direct interference from environmental factors.

The advent of molecular technologies resulted in the exploitation of molecular markers for diversity studies. Taking *Oryza sativa* as an example, diversity indices and the pattern of diversity in sets of germplasm have been assessd using isozymes and RFLP data [37]. In maize, markers such as RAPDs, SSRs and AFLP are used [38].

2.6.5. Use of Molecular Markers to Identify Distant Parents in Hybrid Development

Heterotic groups are the backbone of successful hybrid breeding. Molecular markers are used to classify parental lines into different heterotic groups. This reveals genetic diversity at the whole genome level, and helps identify effects of selection, genetic drift, and mutation.

Marker-based groupings reflect the genetic differences among parental lines. They can utilize for effective selection of heterotic hybrid parents. In general, heterotic groups constructed on the basis of marker information match up very well with pedigrees, but missing historical information, such as the incomplete pedigree information or ambiguous pedigree, will not affect the marker-based method.

In maize, different types of molecular markers have been successfully used to differentiate heterotic groups with results that are consistent with pedigree-based grouping [39]. Based on heterosis and combining ability analyses using cultivars from different heterotic groups, Peng *et al*. (1998) proposed seven heterotic patterns for the utilization of maize heterosis. Divergence at molecular marker loci has been useful in assigning maize inbred to known heterotic groups previously established in breeding programs and the molecular information agreed with pedigree information [35].

Zhang *et al [37]* studied molecular divergence and hybrid performance of rice using two types of molecular markers, RFLPs and AFLPs. Their results suggested the existence of two heterotic groups within *indica*. Mackill *et al* [40], obtained similar grouping results using RAPD and SSR markers, Xiao *et al*. [41] separated ten parental lines into two major groups that correspond to *indica* and *japonica* subspecies. These researches indicated that molecular markers are useful tools in detection of genetic diversity between parental cultivars.

3. Materials and Methods

This study was conducted at Biotechnology division, Plant Genetic Resources Centre (PGRC), Gannoruwa.

3.1. Plant Materials

Twenty three maize pure lines including local lines and CIMMYT QPM lines and CIMMYT normal lines were selected for the study (Table 4). Seeds of those accessions, which were obtained from the Gene Bank of Plant Genetic Resources Centre, Gannoruwa and Field Crop Research Institute at Mahailuppallama were used for this study.

Table 4. List of The Inbred Lines Used In The Study

	Inbred Lines	Name
1	CIMMYT normal old lines	CML 20
2		CML 348
3		CML 322
4		CML 326
5		CML 338
6		CML 451
7	CIMMYT normal new lines	CLO 2450
8		CLRCY 017
9		CLQRCYQ 49
10		CLQRCYQ 59
11		CLQRCYQ 71
12	CIMMYT QPM lines	CML 164
13		CML168
14		CML 171
15		CML 189
16		CML 193
17		CML 194
18	Local inbred lines	No 11- Red
19		No 20- Yellow
20		No 21-Red
21		No 29- Yellow
22		No 44
23		Rohini

3.2. DNA Extraction

DNA was extracted from 14 days old immature leaves of maize using modified CTAB method following the protocol optimized by Biotechnology Division, Plant Genetic Resources Center, Gannoruwa, Sri Lanka.

Homogenization: Fresh, immature maize leaves were cut into small pieces and approximately 1g was obtained. These pieces of immature maize leaves were crushed into a fine powder in liquid nitrogen using a clean mortar and pestle. The grinding process was done rapidly to avoid thawing of leaves.

Lysis: The ground material was suspended in a 1.5 mL micro-centrifuge tube containing 600 µL of pre-warmed (65 ºC) 2% CTAB extraction buffer [2% CTAB (Cetyltrimethyl ammonium bromide), 1M NaCl (Sodium chloride), 0.5M EDTA (pH 8.0) (Ethylene diamine Tetra Acetic acid), 1M Tris - HCl (pH 8.0)], in a water bath (HI TEC-BT 47), and 1.2 µL of 1% β - mercaptoethanol was added to prevent accumulation of polyphenols in the ground materials. The powder of plant material, extraction buffer and β - mercaptoethanol were mixed thoroughly and incubated in a water bath with shaker (HI TEC-BT 47) at 65°C for 30 minutes.

Chloroform extraction: After the incubation, samples were kept for cooling and 600 µL of Chloroform / Isoamyl alcohol mixture (24 Chloroform: 1 Isoamyl alcohol v/v) was added to each tube, inverted and shaken well for 10 minutes. The tubes were centrifuged at 11,000 rpm for 10 minutes at 4°C temperature using a bench top centrifuge (Mikro 22R). The aqueous supernatant was carefully transferred to a sterile 1.5 mL micro centrifuge tube using a micropipette. 10% CTAB buffer and 600 µL of Chloroform / Isoamyl alcohol mixture was added to each tube and inverted for about 5 minutes inside the fume hood until a creamy color developed in the solution inside the centrifuge tubes. Then tubes were centrifuged at 11,000 rpm for 10 minutes at 4°C temperature using the bench top centrifuge (Mikro 22R) and the supernatant was separated in to another sterile 1.5 mL centrifuge tube carefully without disturbing interface between supernatant and the plant debris. The Chloroform extraction was repeated by the application of Chloroform / Isoamyl alcohol mixture and subsequent centrifugation at 11,000 rpm for 10 minutes.

DNA precipitation: 0.6 volume of pre-cooled (-20°C) Iso-propanol was added to the tube containing the supernatant obtained by the second Chloroform extraction. The tubes were then inverted gently till DNA gets precipitated, which was visualized in the form of entangled strings. Next, the tubes were stored at -20°C for overnight to increase the yield of nucleic acid.

The resultant DNA was obtained in pellet form by centrifugation at 11,000 rpm for 10 minutes. The Supernatant was discarded and the DNA pellet was washed with 70% ethanol by shaking and kept for 1hour. Then remaining 70% ethanol in the form of supernatant was evaporated and tubes were placed on a paper towel with the cap open for the DNA pellet to be dried overnight at room temperature.

Storage of DNA: The pellet was dissolved in 50 µL of TE buffer [1M Tris - HCl (pH 8), 0.5M EDTA (pH 8.0) (Ethylene diamine Tetra Acetic acid)]. Afterwards it was stored at -20°C until further use.

3.3. RNAse Treatment

Dissolved DNA was treated with RNAse [10 mg/ml RNAse A, 0.01M Sodium acetate (pH 5.2)]. Then treatment was done according to the amount of TE used to dissolve the DNA pellet. 5 µL of RNAse was treated for 100 µL of dissolved DNA.

3.4. Quantification of DNA

Extracted DNA samples were quantified by comparing the band intensity of known DNA quantity through gel electrophoresis. Two different concentrations of uncleaved λ DNA (λ_{25} and λ_{50}) were used.

Agarose gel electrophoresis

Electrophoresis gel casting tray was cleaned well using 70% alcohol and sealed well using thick tapes. Compatible combs were inserted and the plate was leveled using a standard level. At the same time 1.3 g of agarose was measured and 130 mL of 0.5x TBE buffer was

prepared [5× Tris - Base 54 g, Boric Acid 27 g, 0.5M EDTA (pH 8.0)] to make 1% agarose and added into 500 mL titration flask. This mixture was dissolved using microwave oven (National - NN-S 551 F) and the completely dissolved solution was allowed to cool and poured into the gel casting tray before solidify. After 20 minutes, combs and tapes were removed and the gel casting tray with the gel was dipped in the buffer tank (BIORAD) containing 0.5× TBE buffer. The quantity of the extracted DNA was determined by running 5 µL of each extracted DNA sample, mixed with 2 µL of DNA loading dye (60% glycerol, 10 mL of 1× TBE, 0.025% xylene cyanol, 0.025% bromophenol blue and sterile distilled water for preparation of 100 mL). Five micro liters (5µL) of uncleaved λ DNA (λ_{25}, λ_{50}) was loaded for quantification. Electrophoresis was done for nearly one and half hours under 70V using the power supply (CONSORT-E 833) (Figure 1). The gel was stained for 10 minutes in Ethidium Bromide (0.5µL/mL) solution. After staining, the gel was de-stained for 5 minutes using a flowing water bath. DNA bands were visualized under UV light in Gel documentation system (BIO RAD).

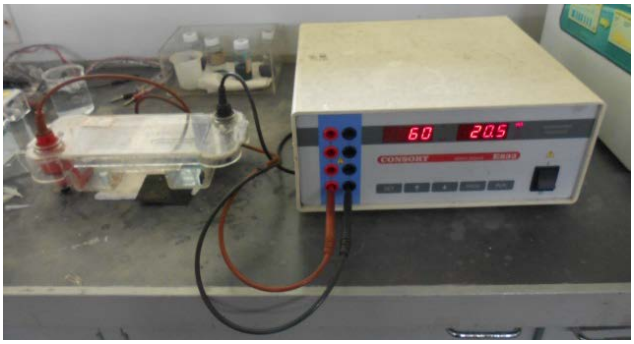


Figure 1. Agarose gel electrophoresis apparatus

3.5. Polymerase Chain Reaction (PCR) Amplification

SSR primers used for molecular analysis

Four SSR primers (Table 5) were used for the detection of genetic distance among maize inbred lines.

Table 5. Primer Details

	Primer	Primer sequence	Annealing temperature
1	*umc* 1023	5' CTTGTGCCACCACATGCAGTA 5' CAGTTTGGAACAGGGAAAAGT	60^0C
2	*Phi* 402893	5' CATCCCCCTGCTGCTGCTG 5' AGGGGATCATGTGCCGAAGGCC	60^0C
3	*Phi* 112	5' TCGTAATTGTTTGTCATGTTGC 5' TCATTGTAGTGAGGTGCTAGTG	58^0C
4	*Phi* 041	5' TTGGCTCCCAGCGCCGCAAA 5' GATCCAGAGCGATTTGACGGC	55^0C

PCR was done using Thermal cycler programme for maize optimized at Biotechnology Division, Plant Genetic Resources Centre, Gannoruwa. 15 µL PCR reaction mixtures was prepared using 10 ηg/µL concentrated DNA of 23 maize samples, PCR buffer, $MgCl_2$, dNTP, forward and reverse primers, sterilize distilled water and *taq* DNA polymerase enzyme (Table 6).

Table 6. Composition of 15µl of PCR Master Mixture

Chemical ingredient	Amount added for 15µL PCR reaction mixture (µL)	Final concentration
5× PCR buffer	3.0	1×
10mM dNTP	0.3	0.2 mM
25mM $MgCl_2$	1.5	2.5 mM
20µM forward primer	2.0	2.7 µM
20µM reversed primer	2.0	2.7 µM
5 U / µL *taq* DNA polymerase	0.2	1unit
10ηg/µL DNA template	6.00	60 ηg

First, 200 µL thin wall sterilized PCR tubes were taken and labeled correctly and 6 µL of template DNA was added into each tube. Master mixture was prepared by adding, 5x PCR buffer, $MgCl_2$, dNTP mixture, forward primer, reverse primer, and taq DNA polymerase. Master mixture was mixed with a short vortex and 9 µL of prepared PCR mixture was added in to each PCR tube that contained DNA templates and the lid was closed well to avoid evaporation during amplification. After giving a short vortex (VOTEX- GENIE 2) and quick spin (BIOFUGE PICO), tubes were placed in the Thermal cycler.

Each PCR amplification consisted of an initial denaturation step at 94°C for 4 minutes followed by 30 cycles consisted of 3 steps, denaturation step at 94°C for 1 minute, annealing step for 1 minute in which the specific annealing temperature for each primer was provided and an extension step at 72°C for 2 minutes. At the end of the final cycle, final extension was carried out at a temperature of 72°C for 7 minutes, with subsequent holding temperature at 4°C. PCR was carried out in Eppendorf-Mastercycler gradient, thermal cycler (Figure 2(a)).

3.6. Confirmation of PCR Products

Amplified products were observed using 1.5% agarose gel electrophoresis. The gel was under went in 0.5 x TBE at 60 V for 1 hour and 30 minutes. After the electrophoresis the gel was stained with Ethidium Bromide according to the procedure followed in DNA quantification. DNA bands were detected under UV light and the gel pictures were taken using a gel documentation system (BIORAD) (Figure 2(b)).

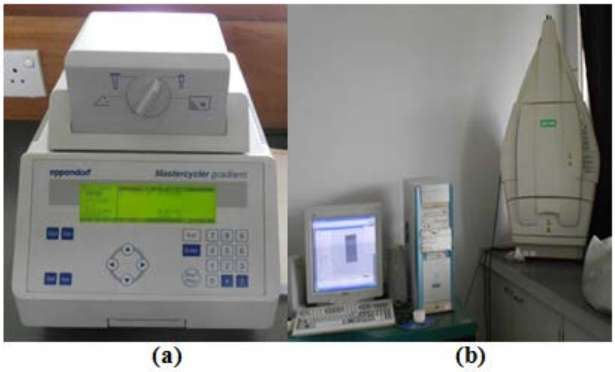


Figure 2. (a) Eppendorf- Mastercycler gradient, thermal cycler, (b) BIORAD gel documentation system

3.7. Resolution of Amplified Products

Polyacrylamide gel electrophoresis

8% Polyacrylamide gel electrophoresis (PAGE) was done for the detection of SSR polymorphism

Table 7. Buffers and Solutions, Required For Polyacrylamide Gel Electrophoresis

Stock solution	Ingredients
30% Acrylamide	29g of Acrylamide, 1g of N-N methylbisacrylamide in 100 mL of deionized water
5x TBE buffer	54g of Tris-base, 27.5g of boric acid, 20mL of 0.5M EDTA in 1L of deionized water
10%Amonium persulfate	1g of Amonium persulfate in 10 mL of deionized water (APS)

Cleaning of glass plates

Glass plates were dipped in potassium hydroxide (KOH)/methanol solution (5 g of KOH pellets in 100 mL of methanol) for 2 hours. Then glass plates and spacers were washed with warm detergent solution and rinsed with tap water and deionized water, finally rinsed with ethanol and kept aside to dry.

Siliconization of glass plates

Glass plates were laid on a pad of paper in a fume hood and a small quantity of siliconizing fluid (sigmacote) was poured onto it. The fluid was wiped over the plates, and allowed to dry. This treatment prevents the gel from sticking tightly to one plate and reduces the possibility of 'gel tear', when the mold is dismantled after electrophoresis.

Assembling of glass plates with spacers

Larger (un-notched) plate was laid on the table and the spacers were arranged at each side parallel to the two edges. The inner (notched) plate was laid in position, to rest on the spacer bars. Plates were clamped together and carefully placed in Gel apparatus.

Preparing the gel solution

Taking into account the size of the glass plates and the thickness of the spacers, volume of gel required was calculated. The gel solution was prepared with the desired polyacrylamide percentage (Table 8), which gives the amount of each component required to make 100 mL. 35 µL of TEMED was added to each 100 mL of acrylamide solution, and the solution was mixed with gentle swirling. Acrylamide was allowed to polymerize for 50-60 minutes at room temperature.

Table 8. Amount of Each Component Required To Make 100 Ml Of 8% Polyacrylamide Gel

Ingredients	Volume (mL)
30% Acrylamide	26.6 mL
5X TBE buffer	20 mL
10% APS	0.7 mL
Water	52.7 mL
TEMED	35µL

Solution was drowned into the barrel of a syringe. Syringe was inverted to expel any air that has entered the barrel. Nozzle of the syringe was introduced into the space between the two glass plates. The acrylamide gel solution was expelled from the syringe, filling the space almost to the top. Appropriate comb was immediately inserted into the gel, taking care not to allow air bubbles get trapped under the teeth. After polymerization is complete, the comb and the top of the gel was surrounded with paper towels, which were soaked in lX TBE until needed. When ready to proceed with electrophoresis, comb was removed carefully and wells were rinsed out with distilled water immediately. Gel plates were attached to the electrophoresis tank with notched plate facing inward toward the buffer reservoir. Electrophoresis tank was filled with 1X TBE buffer. Wells were flushed out with 1X TBE using a pasture pipette.

Loading the samples and running the gels

Five micro liters (5µL) of DNA sample was mixed with the appropriate amount of 6 X gel-loading buffer. Mixture was loaded into the wells, using a micropipette, equipped with a drawn-out plastic tip (Figure 3). Electrodes were connected to a power supply (CONSORT-E 833) and electrophoresis was done at 120V until the marker dyes migrated the desired distance ($3/4^{th}$ of the length of the glass plate). Glass plates were detached and the upper plate was pulled smoothly away. Gel was detached from the lower plate by flushing with distilled water through the gel and the plate. The gel was kept on a wrapping paper.

Figure 3. Polyacrylamide gel electrophoresis apparatus

Staining of gels and visualization

The gel was gently stained with Ethidium Bromide (0.5µg/ml) solution. After staining, the gel was de-stained for 20 minutes, using distilled water bath. Bands were visualized under UV light in the Gel documentation system (BIORAD).

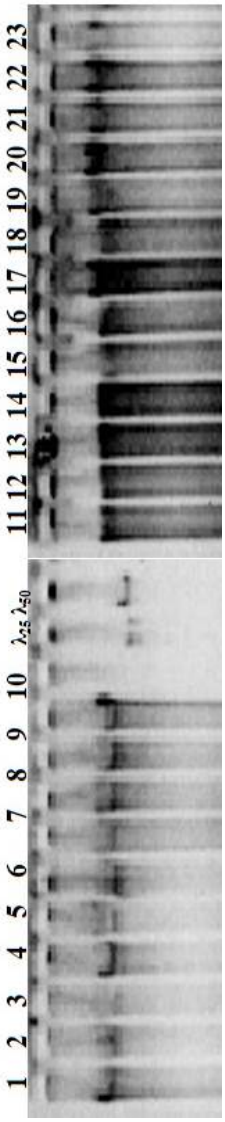
3.8. Data Scoring and Analysis

The Ethidium Bromide stained polyacrylamide gels were manually scored by visual observations of presence (1) and absence (0) of bands. Using these data genetic diversity parameters were computed based on SSR data using the POWERMARKER Version 3.1 [42]. Major allele frequency, genotype frequency, gene diversity and polymorphism information content (PIC) values were calculated. Genetic distances were estimated based on Nei's distance (Nei's, 1972) which facilitates the identification of similarities and dissimilarities among

selected maize inbred lines and finally the phylogenetic tree was constructed to elucidate the relationship among selected maize inbred lines.

4. Results and Discussion

4.1. DNA Extraction and Quantification



1-CML 20, **2**-CML 348, **3**-CML 322, **4**-CML 326, **5**-CML-338, **6**-CML 164, **7**-CML 168, **8**-CML 171, **9**-CML 189, **10**-CML-193, **11**-CML 194, **12**-No11, **13**-No20, **14**-No21, **15**-No29, **16**-No44, **17**-Rohini, **18**-CML 451, **19**-CLO2450, **20**-CLRCY017, **21**-CLQRCYQ49, **22**-CLQRCYQ59, **23**-CLQRCYQ71

Figure 4. 1% agarose gel image of the raw DNA of tested maize inbred lines

About 14 days old, maize seedlings, grown under the plant house condition were used for the extraction of DNA. CTAB (Cetyl Trimethyl Amonium Bromide) method which had been slightly modified by the Plant Genetic Resource Centre, Gannoruwa, Sri Lanka was used for the extraction of DNA. Immature leaf materials were used in order to give good quality DNA. The presence of DNA was confirmed by running in an agarose gel with 25 and 50 undigested λ DNA, and stained with Ethidium Bromide (Figure 4).

4.2. PCR Amplification of the Samples

23 inbred lines were amplified using 4 SSR primers. Amplified PCR products were confirmed by 1.5% agarose gel electrophoresis. Figure 5 shows the amplified products of *umc 1023* and *phi 402893* in 1.5% agarose gel. All 4 SSR primers confirmed the amplification in 1.5% agarose gel, when visualized under UV light in Bio Rad gel documentation system.

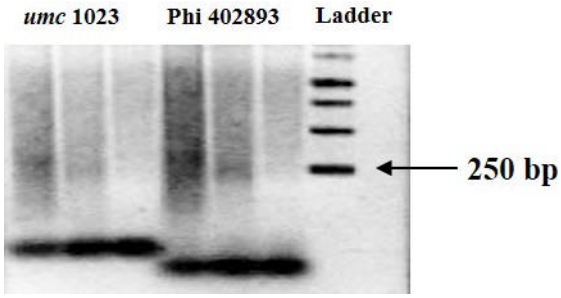


Figure 5. PCR confirmations of *umc*1023 and *phi* 402893 in 1.5% agarose gel

4.3. Resolution under Polyacrylamide Gel Electrophoresis

A total of four SSR primers were screened to identify polymorphism in all 23 inbred lines. The amplified PCR products were electrophoresed on 8% polyacrylamide gel and visualized under UV light after staining with Ethidium Bromide. The banding patterns were observed.

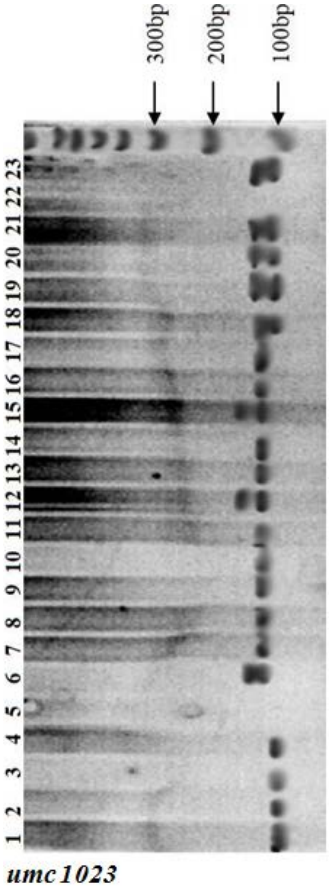
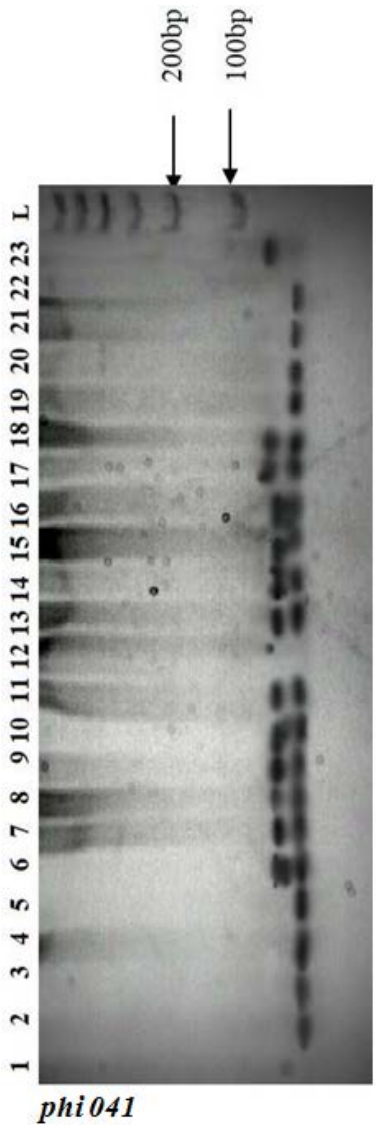


Figure 6. A Resolution of PCR products of 23 inbred lines in 8% polyacrylamide gel; (gel image of *umc 1023*)

umc 1023 has shown good polymorphic banding pattern with inbred lines which could be more informative to the distance and diversity analysis (Figure 6 A). CML 338 and CLQRCYQ 59 did not show any bands in the PAGE. It may be due to null allele or poor PCR amplification.



1-CML 20, **2**-CML 348, **3**-CML 322, **4**-CML 326, **5**-CML-338, **6**-CML 164, **7**-CML 168, **8**-CML 171, **9**-CML 189, **10**-CML-193, **11**-CML 194, **12**-No11, **13**-No20, **14**-No21, **15**-No29, **16**-No44, **17**-Rohini, **18**-CML 451, **19**-CLO2450, **20**-CLRCY017, **21**-CLQRCYQ49, **22**-CLQRCYQ59, **23**-CLQRCYQ71
CML 20, CML 168, CML 189, No-29, Rohini CLRCY 017 and phi 112 showed polymorphic bands except CML 332 and No-20 in *Phi 402893*

Figure 6.B Resolution of PCR products of 23 inbred lines in 8% polyacrylamide gel; (gel image of *phi 041*.)

Polymorphic banding pattern was shown for primer *phi 041* (Figure 6 B), except CML 20, No-11 and CLQRCYQ 59. The other samples displayed bands which revealed genetic variation.

4.4. Diversity Analysis

The genotypic data obtained from amplified 23 maize inbred lines by PCR with different primers were analyzed using POWERMARKER V3.1. It was mainly used to obtain diversity parameters (Table 9), genetic distance matrix (Table 10) and the Phylogenetic Tree (Figure 7).

Table 9. Diversity Parameters Of Tested Maize Inbred Lines

Marker	Major Allele Frquency	Allele No	Gene Diversity	Hetero zygosity	PIC*
umc1023	0.72	3.00	0.44	0.08	0.39
phi402893	0.70	3.00	0.46	0.33	0.42
phi112	0.83	2.00	0.28	0.24	0.24
phi041	0.70	2.00	0.42	0.46	0.33
Mean	0.74	2.50	0.40	0.28	0.34

* PIC-Polymophic Information Content.

In Table 9, the allele number provides the number of alleles obtained from each primer used in the analysis. The maximum numbers of alleles have been produced with the primers *umc 1023* and *phi 402893* with each amplifying 3 alleles. *Phi 112* and *phi 041* were able to amplify 2 alleles in the genome of the maize inbred lines used in the analysis. Mean allele number of tested population was 2.5 with all the tested four primers. Polymorphic information content (PIC) ranged from 0.24 in *phi112* to 0.42 in *phi402893*. *umc 1023* and *phi 041* gave 0.39 and 0.33 PIC values respectively. Gene diversity varied from 0.28 (*phi112*) to 0.46 (*phi402893*). *umc 1023* and *phi 041* gave 0.44 and 0.42gene diversity values respectively.

By using diversity parameters (Table 9), allele frequencies can be considered as the basic genetic parameter of a population, both in the sense that populations with different genotype frequencies may have Identical allele frequencies, and that incident has been defined as a change in allele frequencies through the time [46]. Major allele frequency provides values for the frequency of the alleles amplified in each individual within the population concerned, with respect to each primer. In situation where a less number of alleles are presented by a particular primer across the population, the allele diversity is less, hence the alleles which are present, come in higher frequencies. Primers *phi 112* and *phi 041* having the least number of alleles amplified have recorded the higher major allele frequency while primer *phi402893* which has the highest allele numbers has recorded the least major allele frequencies.

Gene diversity has negative correlation with the major allele frequency. The primer which gives the highest major allele frequencies shows the least gene diversity, while those have lower major allele frequencies show higher gene diversity. As an example, the primer *phi 112* possesses the lowest number of alleles, i.e. 2 alleles, showing the highest major allele frequency of 0.83 and the least gene diversity 0.27. As well as the primer *phi 402893* and *umc 1023* shows the greatest number of alleles that have the highest gene diversity of 0.47 and 0.44 respectively. This phenomenon is a result of the number of alleles amplified by each primer. When the numbers of alleles are less, the genetic variation within the population is less with respect to specific primer.

The heterozygosity provides values to get an idea about the amount of heterozygous alleles amplified by the specific primer. As an example primer *phi 041* has the highest heterozygosity of 0.46. This shows that approximately 20% of alleles amplified by particular primer can be heterozygous alleles.

Table 10. Nei's genetic distance matrix developed for the selected maize inbred lines

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	0.0000																						
2	0.1464	0.0000																					
3	0.0000	0.0976	0.0000																				
4	0.0000	0.1464	0.0000	0.0000																			
5	0.0000	0.0976	0.1464	0.0976	0.0000																		
6	0.1838	0.2383	0.1225	0.0919	0.0976	0.0000																	
7	0.6464	0.7643	0.6464	0.5286	0.2929	0.2704	0.0000																
8	0.6464	0.3964	0.4310	0.3964	0.2929	0.2028	0.3333	0.0000															
9	0.6464	0.4310	0.6464	0.5286	0.2929	0.2704	0.3333	0.0000	0.0000														
10	0.6464	0.5732	0.7643	0.6464	0.5286	0.4528	0.3333	0.2500	0.0000	0.0000													
11	0.6464	0.3427	0.3593	0.3427	0.2213	0.1491	0.3581	0.0186	0.0247	0.2686	0.0000												
12	0.6464	0.6667	1.0000	0.7643	0.6464	0.5483	0.5817	0.3878	0.0817	0.0544	0.3878	0.0000											
13	1.0000	0.4085	0.5061	0.5061	0.1127	0.2479	0.0025	0.0993	0.0025	0.3350	0.1113	0.5817	0.0000										
14	0.6464	0.3064	0.5061	0.4528	0.1728	0.2591	0.3350	0.0745	0.0017	0.2513	0.0835	0.3878	0.0000	0.0000									
15	0.6464	0.4431	0.6646	0.5407	0.3110	0.3581	0.4314	0.0980	0.0980	0.0980	0.1291	0.0105	0.1521	0.1014	0.0000								
16	0.6464	0.3232	0.5286	0.4697	0.1953	0.2760	0.3333	0.0732	0.0000	0.2500	0.0918	0.3878	0.0017	0.0013	0.0980	0.0000							
17	0.6464	0.4431	0.6646	0.5407	0.3110	0.2825	0.3338	0.0004	0.0004	0.0004	0.0314	0.0817	0.0057	0.0038	0.0976	0.0004	0.0000						
18	0.6464	0.4431	0.6646	0.5407	0.3110	0.2825	0.3338	0.0004	0.0004	0.0004	0.0314	0.0817	0.0057	0.0038	0.0976	0.0004	0.0000	0.0000					
19	0.6464	0.3232	0.3333	0.3232	0.1953	0.1296	0.4310	0.0732	0.0976	0.3232	0.0195	0.3878	0.1728	0.1296	0.2074	0.1464	0.1097	0.1097	0.0000				
20	0.6464	0.3333	0.5000	0.4310	0.1464	0.1728	0.4310	0.0976	0.0976	0.0976	0.0260	0.0817	0.1127	0.0751	0.2074	0.0976	0.1097	0.1097	0.0000	0.0000			
21	0.6464	0.3232	0.3333	0.3232	0.1953	0.1296	0.4310	0.0732	0.0976	0.3232	0.0195	0.3878	0.1728	0.1296	0.2074	0.1464	0.1097	0.1097	0.0000	0.0000	0.0000		
22	0.6464	0.4310	0.5000	0.4310	0.2929	0.4310	1.0000	0.3333	0.5000	0.6667	0.3333	0.4841	0.6464	0.4310	0.1464	0.4310	0.5000	0.5000	0.5000	0.5000	0.3333	0.0000	
23	0.5000	0.5286	0.6464	0.4310	0.1464	0.1728	0.0976	0.0976	0.0976	0.0976	0.1224	0.2281	0.0025	0.0993	0.1957	0.0976	0.0980	0.0980	0.1953	0.1953	0.1953	0.6464	0.0000

The relative informativeness of each marker can be evaluated based on its PIC value, which is a function of the number of alleles and allele frequencies at any given locus. This reflects the amount of polymorphism

The highest PIC value of 0.42, obtained by the primer *phi 402893* which has an allelic number of 3 and also this primer showed the highest gene diversity among the population. Although, *umc1023* primers has the same allele number. The PIC values which has obtained are different. This could be due to di-allele frequency, the degree of polymorphism revealed by these primers being different to each other, even though they have same allelic number. Sorajjapinun (2012) reviewed that the PIC values of maize in polymorphic SSR markers were high, ranging from 0.04-0.90 (mean value 0.67), indicating that SSR markers could be efficiently applied to detect polymorphism even with a relatively low number of primers.

4.5. Genetic Distance and Phylogenetic Analysis

4.5.1. Genetic Distance

Genetic distances between the selected maize inbred lines used for the analysis were calculated using Nei's genetic distance matrix (Table 10). The distance ranged from 0.0000 to 1.0000. It was a clear evident that the highest genetic distance of 1.00 was shown by CML 20 with No-20, No-11 with CML 322 and CLQRCYQ 59 with CML 168.Those are the most divergent pairs in this study. The highest genetic divergence was exhibited between CIMMYT normal old lines and local lines, CIMMYT normal new lines and CIMMYT QPM lines, respectively.

The second highest genetic distance was showed as 0.7643 between two pairs, CML 168 and CML 348, No-11 and CML 326.

CML 20 which is CIMMYT normal old line showed genetic distance of 0.0000 with CML 322, CML 326 and CML 338s except CML 348 with the genetic distance of 0.1464.

CML 189 demonstrated a genetic distance of 0.000 with CML 171, CML 193 and No-44.Rohini and CML 451 showed a genetic distance of 0.004 with CML 171, CML 189, CML 193 and No-44.Rohini and CML 451, No-20 and No-21 showed the genetic distance of 0.0000with each other respectively. In CIMMYT normal old lines, highest genetic distance of 0.1464 was showcased between CML 20 and CML 348, CML-348 and CML 326, CML 322 and CML 338.

Among CIMMYT QPM lines, the highest genetic distance of 0.4528 was shown by CML 164 and CML 194.0.5817 is the highest genetic distance shown between local lines, NO-11 and No-20. Among CIMMYT normal new line CLQRCYQ 59 showed the highest genetic distance of 0.500 with CML 451 and CLO 2450. In local lines the lowest genetic distance of 0 was shown by No-20 with No-21 and the highest genetic distance of 0.5817 was shown by No-11 and No-20. The second highest genetic distance value of 0.3900 was shown by No-11 with No-44 and No-11 with No-21, and all other inbred lines comprised of comparatively low genetic distance value with each other.

4.5.2. Phylogenetic Tree

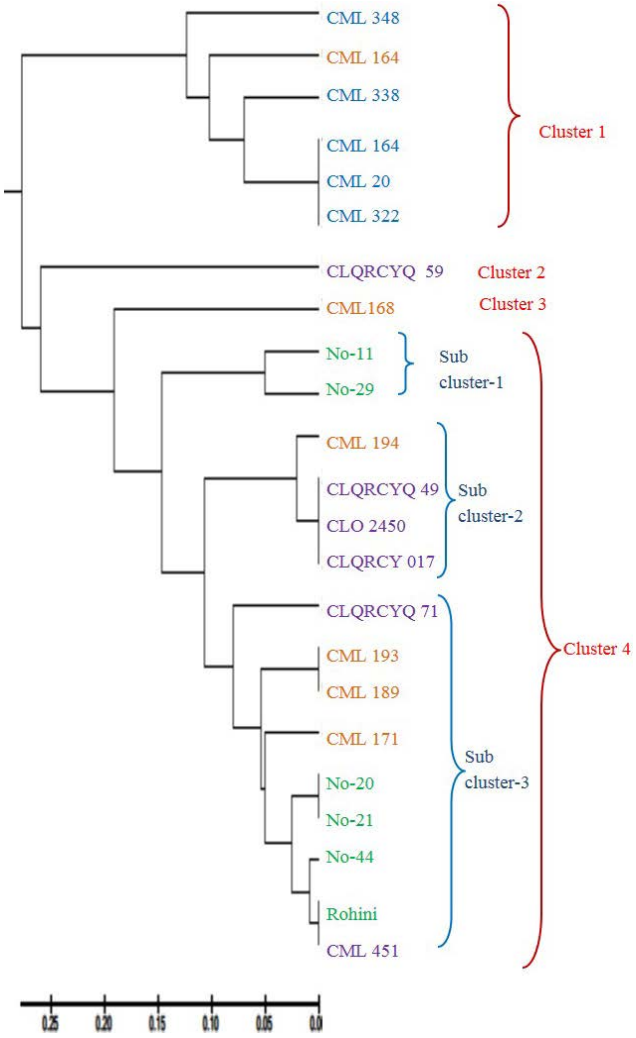


Figure 7. Phylogenic tree of tested maize inbred lines based on SSR data, using Nei's genetic distances and UPGMA algorithm

A phylogenetic tree was constructed using Nei's genetic data and UPGMA algorithm derived from the analysis of maize inbred lines, using SSR markers (Figure 7). It was figured out that all 23 inbred lines were grouped into four major clusters.

Six inbred lines were grouped in the first major cluster which has more than 25% genetic difference from the rest of the inbred lines. All these six inbred lines had been developed at CIMMYT (CML 20, CML 322, CML 348, CML 326, CML 338 and one, CIMMYT QPM line; CML 164).

CIMMYT normal new inbred line, CLQRCYQ 59 was not grouped with any other lines and stayed separately. QPM line CML 168 made the third cluster, which is genetically distinct from the rest of the inbred lines.

The fourth major cluster consisted of more than 60% of the tested inbred lines. All locally developed inbred lines, some of the QPM lines and new improved inbred lines were grouped under this cluster. It could be grouped into three sub clusters. Two local inbred lines No-11 and No-29 were grouped into one sub cluster. CIMMYT new inbred lines CLQRCYQ 49, CLO 2450 and CLQRCY 017, and QPM line; CML 194 were grouped into second sub cluster.

Third sub cluster comprised, Local lines; NO-20, NO-21, NO-44 and Rohini, CIMMYT normal lines CLQRCYQ 17 and CML 451, CML 193, CML 189 and CML 171 which come under CIMMYT QPM lines. CML 451 which is CIMMYT line was grouped along with local inbred lines with low genetic difference. Among them CML 451 and Rohini had very low genetic difference.

Nei's genetic distance and UPGMA clustering pattern based on SSR analysis revealed that there is a considerable genetic diversity among tested maize inbred lines. The resulted clustering pattern of SSR analysis revealed that lines belonging to same cluster have low genetic difference and inbred lines in different clusters has considerable genetic distance.

According to the Table 10, maximum genetic distance (1.0000) was observed between CML 20 and No-20, No-11 and CML 322, CLQRCYQ 59 and CML 168 Furthermore, when comparing the genetic distances as in the matrix. CML 20 which is a CIMMYT normal old line shows zero genetic distance with other normal lines except CML 348 This means CIMMYT normal old lines share same genetic makeup. But they showed high dissimilarity in the genetic makeup with other inbred lines. In the phylogenetic tree, all CIMMYT normal old lines are in cluster 1 at genetic distance of 0.25.Among them CML 20 shows a genetic distance of 0.0 with CML 322 and CML 326.They were grouped in same mini cluster. Other two normal lines named as CML 348 and CML 338 got grouped in two separate mini clusters with relatively low genetic difference. But in the genetic distance matrix CML 338 shared a genetic distances of 0.000 with CML 20.

CML 189 can have same genetic makeup with CML 171, CML 193 and No-44. Rohini and CML 451 can share same genetic makeup with each other as well as with CML 171, CML 189, CML 193 and No-44. No-20 and No-21 also have similar genetic makeup.

According to Wasala *et al* [43], even though the genetic distances are 0.000, there can be some significant differences in morphological level. Therefore, it is hard to establish a statement about the distance of particular accessions, using only the results of molecular study and thus need to consider the geographical and morphological characters as well.

Among CIMMYT normal new lines, CLQRCYQ 59 and QPM line; CML 168 comprised of two separate major clusters. Both of these lines showed higher genetic distance of 1.000 between each other. This result showed these two exotic inbred lines share low genetic similarity.

Considering the local lines selected for the study. No-11 and No-20 showed higher genetic distance of 1.0000 with exotic lines. According to the phylogenetic tree No-11 and No-29 were grouped in one sub cluster indicating similarity between the two lines. These two lines shared higher dissimilarity from others. No-20, No-21, No-44 and Rohini got grouped in one cluster sharing same alleles. Among them No-20 and No-21 shared minimum genetic distance and this pair shows the lowest genetic distance among the local lines which is 0.000. No-44 was grouped in a separate cluster. Rohini and CML 451 were grouped in another cluster. This result showed CML 451 which is CIMMYT normal new line shared same alleles as local inbred lines and have high relationship between the two groups. Magorokosho [44] reported two main reasons for this high relationships; common parentage or ancestry of the two groups, and *creolization* between inbred lines.

When consider the CIMMYT QPM line, QPM inbred lines were grouped in several clusters. CML 164 got grouped to first major cluster along with normal lines. CML 168 was grouped individually in the third cluster showing higher genetic dissimilarity among other QPM lines. CML 194, CML 193, CML 189 and CML 171 were grouped in the fourth cluster. Among them CML 193, CML 189 and CML 171 were grouped in the third sub cluster with low genetic distance. CML 164 which was grouped in the first major cluster shows genetic distances of 0.2704 with CML 168and CML 189, 0.2028 with CML 171 and a distance of 0.4528 and 0.1491 with CML 193 and CML 194 respectively. But according to genetic distance matrix, the highest genetic distance among QPM lines was demonstrated in CML 164 and CML 193 with a genetic distance of 0.4528. This results show QPM lines are different from other lines because of their modified gene although same genetic makeup is shared with other inbred lines.

4.6. General Discussion

Information about the genetic diversity of inbred lines is essential in selecting parents for maize hybrid breeding programmes. Utilization of diverse parents in the process of hybridization has the greatest influence on producing high yielding hybrids.

Genetic difference between the inbred or pure line parents presumably contributes to the genetic basis of heterosis. Farooq and Azam [30] reviewed the uses of molecular markers in measuring genetic distance and heterosis in plant breeding.

Maize is diploid cross pollinated species with great phenotypic and genotypic diversity. But the intensive artificial selection significantly narrowed the genetic basis of maize germplasm resulting that the majority of hybrids grown today derive from a few inbred lines [27].

The markers divided the germplasm into distinct sets according to their genetic distances and origins. Their information can be utilized to compare the performance of hybrids derived from the parents with different degrees of genetic distance. The heterotic patterns detected from various parental lines will be useful to the plant breeders to decide cross combinations for promisinghybrids. This approach will help to reduce the number of poor performing hybrids entered into yield trials.

Several methods have been developed to predict hybrid performance in maize using genetic markers. Reif *et al.*, [45] has given information about molecular markers which were used to assign new germplasm to heterotic pools in maize. Liu and Wu (1998) showed that SSR marker technology could be used to identify heterotic patterns of the parental lines in hybrid rice production.

SSR markers usually reveal single-copy, homozygous loci and allelic heterogeneity is rare in pure line varieties. These facts simplified the work of SSR for the analysis of genetic diversity of maize varieties. The genetic diversity of maize has been studied using several methods such as their morphological and physiological characters, isozymes,

RFLP markers and microsatellite markers. Out of these SSRs are highly informative PCR-based markers. Reif *et al.*, 2005 [47].

SSR markers proved to be a useful tool for clarifying the genetic diversity among different plant varieties. But when a genetic diversity analysis is carried out, it is essential to select SSR markers which represent the total genome of selected accessions, because a molecular characterization study should have the ability to reveal the maximum genetic variation or genetic relatedness of a population [46]. The use of considerable numbers of primers to amplify substantial amount of the genome is very important for a reliable and informative genetic diversity study.

The number of primers used in the current study is not enough to get more reliable and informative results. More SSR primers to represent whole maize genome is needed to make a precise conclusion about the genetic distances within the selected accessions.

The QPM lines selected for the study have clustered in different groups and showed dissimilarities in genetic level. This can be witnessed by the phylogenetic tree derived in this study (Figure 7). Findings of this study highlight that even though labeled as QPM inbred lines, a genetic diversity analysis is essential to identify the distinct parents.

When analyzing the genetic distance, molecular markers are identified as useful tool in analyzing the genetic distance in plant population at DNA level. Molecular markers are also helpful in understanding the phylogenetic relationships among plant varieties within a given taxonomic group. Even duplicate accessions can also be distinguished using molecular markers [46].

Comparison of the results of the cluster analysis among maize inbred lines used in the study revealed that, selected CIMMYT normal lines were significantly different from other lines. CLQRCYQ 59 and CML 168 inbred lines were also different from other inbred lines as well as among each other. Rief *et al.*, [45] reported that genetic relationships indicated by cluster analysis of SSR data can be useful for superior line development from a population created by crossing genetically diverse inbred lines from the same heterotic group. Inbred lines from genetically distant clusters have higher probability to express better specific combining ability and their crossing could maximize the yield of hybrid [46].

These molecular data can be combined with morphological and agronomic data to get a clear picture of the distance among the inbred parents. Therefore, higher hybrid vigor can be forecasted by a cross between those inbred lines in the above mentioned clusters. Using SSR markers it was possible to identify the parental lines with promising heterotic patterns rather than evaluating the whole set of germplasm in more expensive field trials. These results clearly depicts that the variety development cycle can be considerably shortened by the use of molecular markers for parental selection. Hallauer and Malithano [33] showed that crosses of unrelated genotypes contributed to greater yields. Better hybrids can be developed by inbred lines derived from different genetic background. Because of great expenditure of time and money in development, maintenance and evaluation of inbred lines, the application of molecular approach is a valuable tool in early selection of inbred parents.

5. Conclusion

All CIMMYT normal old lines (CML 20, CML 322, CML 348, CML 326, and CML 338) got grouped together and have the least genetic diversity. Local inbred lines showed similar genetic makeup which was distinct from CIMMIT lines. CIMMIT QPM lines scattered throughout and have diverse genetic makeup. CLQRCYQ 59 and CML 168 inbred lines showed distinct genotypes contrast to other inbred lines.

The higher genetic distance between maize inbred lines expressed the distance between parents. Genetic distances which derived from molecular marker data are useful to classify the parents into distinct heterotic groups.

The genetic distances based on marker information proved to be suitable for estimating genetic diversity relationships among tested inbred lines. Moreover, the primers *umc 1023*, *phi 402893*, *phi 112* and *phi 041* can be used to identify the diversity of maize inbred lines.

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