MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF MAIZE INBRED LINES IN KENYA USING SIMPLE SEQUENCE REPEATS

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DECLARATION

I declare that this thesis, which I submit for the degree of Master of Science in Genetics, is my original work and has not been presented for a ward of degree in any University.

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DEDICATION

To two very special people who have always had my interests at heart: Grand Mum and Mum.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
3 BME	β-mercaptoethanol
bp	Base pair
BSA	Bulk Segregant Analysis
CA	Cluster analysis
CIMMYT	International Maize and Wheat Improvement Center
CML	CIMMYT Maize line
CTAB	Cetyltrimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	The mixture of four deoxynucleotides triphosphate
DTS	Days to silking
DTT	Days to tasseling
ERD	Ear diameter
ERH	Ear height
ERL	Ear length
EM	Embu
GS	Genetic similarity
KARI	Kenya Agricultural Research Institute
MAS	Marker Assisted Selection
MALS	Meters above sea level
OPV	Open Pollinated Variety
OSU	Ohio State University
PCA	Principal Component analysis

PCR	Polymerase Chain Reaction
PLH	Plant height
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RWN	Rows per ear
SDW	Seed weight
SNP	Single nucleotide polymorphism
SSR	Simple Sequence Repeat
SD	Standard deviation of the means
SEM	Standard error of the mean
STS	Sequence Tagged Site
Ton	Tonne
UPGMA	Unweighted Paired Group Method using Arithmetic averages
V	Volt
W	Watt
YD	Yield

ABSTRACT

Maize (*Zea mays* L.) is the main staple food in Kenya with ninety percent of the population depending on it for food and income. Despite its importance, farmers in Kenya harvest less than 2.0 ton/ha compared to world average of 5ton/ha. The low productivity is as result of many factors that relate to on farm production constraints and insufficient knowledge on the genetic source of germplasm used for crop improvement. The main objective of this study was to characterize ten elite maize inbred lines using fourteen simple sequence repeats (SSRs) molecular markers and morphological traits in order to better understand germplasm diversity. The field experiment was carried out at Kenya Agricultural Research Institute (KARI) field station at Muguga (2093 meters above sea level). Maize for morphological characterization were planted using the randomized complete block design and grown for one season. Molecular analysis was carried out using genomic DNA extracted from three weeks old leaves using the modified Cetyltrimethyl-ammonium bromide (CTAB) method at KARI Biotechnology center Nairobi. In total 28 alleles were identified, with a mean of 2.0 alleles per locus. Cluster analysis of morphological traits and SSR markers using unweighted pair group method with arithmetic average (UPGMA) revealed three groups among the inbred lines with distinctive genetic profiles and morphological traits. The phenotypic analysis revealed significant variation with respect to plant and ear height, time to anthesis and silk emergence (50%), anthocyanin coloration and yield. Both morphological and molecular

analysis showed that CML206, CML204 and CML312 are related. Likewise CML202 and CML444 clustered together in both analyses. CML395 and CML 442 were also grouped together in both morphological and molecular analysis. The two KARI lines (EM11-133 and EM12-210) were grouped together suggesting a common ancestry. Molecular analysis showed that the line OSU23i is genetically related to the lines EM11-133 and EM12-210. However, morphological analysis classified OSU23i as unique as it did not cluster with any inbred line. Both molecular and morphological traits were necessary in grouping the ten maize inbred lines. The SSR primers revealed genetic distance (<0.3), which is, a good indication confirming the power of SSR markers to distinguish between closely related inbred lines.

As a result of this study breeders will be able to select putative heterotic parents for hybridization and stratify the breeding programs based on the diverse characterization of the inbred lines such as EM11-133 and CML 204, EM12-210 and CML 395 and CML 202 and CML 204 with a genetic distance of 1.54. On the other hand breeders should avoid crossing lines which are closely related such as CML 206 and CML 312, EM11-133 and CML 395 and CML 444 and CML 442 with genetic distance of 0.154. It will also help in designing sound breeding program and improve management strategy for the ten elite maize lines.

CHAPTER ONE

1.0 Introduction

Maize (*Zea mays l*) is the most important food crop in Kenya with a national production of 2.4 million tones in a total area of 1.6 million hectares, which is far below the potential yield (Gebrekidan *et al.*, 1992). It is ranked third with about 80% of cereal production after wheat and rice in most developing countries (CIMMYT, 2002). Ninety percent of the Kenyan population is dependant in maize for food and source of income. Maize provides over 50% of staple calories (Raemeekers, 2001).

The production of maize as a staple food crop by smallholder farmers plays a vital role in alleviating poverty, income generation and contributing to the local and national economy. It accounts for more than 20% of all agricultural production and 25% of agricultural employment in Kenya. For this reason the country's food security and economy strongly relies on maize production (Moaldm, 1998).

The economy of Kenya is mainly based on agriculture and a loss in the staple crop (maize) due to plant pathogens may be enormous. The main diseases are *turcicum* leaf blight, gray leaf spot (GLS), common rust, head smut and maize streak virus (MSV) while pests are stem borers, common weevils and large grain borer and Striga or witch weed (Evenson and Golln, 2003). The reduction in yield is attributed to both biotic and abiotic constraints.

Improvement strategies are carried to breed maize cultivars exhibiting increased resistance to pests and diseases identified as key constraints in maize-growing ecosystems in Kenya as well as develop varieties with characteristics required by farmers to enhance production. This is the only economic solution towards achieving high maize

yields in disease-prone environments. These improvements have led to substantial gains globally (Evenson and Gollin, 2003). The improvements have come from the continued efforts of plant breeders to develop locally adapted varieties that have the characteristics required by farmers to enhance production. Most of the research work in maize problems carried out in Kenya is done at CIMMYT, KARI, Kenya Seed Company among others.

Most of the modern inbred lines used in breeding programs are second, third or fourth cycle lines that were developed from other inbred lines or from synthetic populations derived from crossing inbred lines (Baker, 1984). Although the older generation inbred lines have retired from hybrid seed production in Kenya, they are still widely used in inbred line development, genetic studies, and as testers (Hallauer *et al*, .2000). The availability of different inbred lines from different sources, both within and outside the country, indicates their continued importance (Mauria *et al*., 2000).

Inbred lines once released are maintained for decades through periodic seed increases in breeding programs and at germplasm repositories. Effects of artificial selection regimes, natural selection in maintenance environments, drift, migration (contamination), and mutations lead to genetic changes (Senior *et al*., 1998). Such genetic changes are influenced by the frequency of regeneration, unintentional out-crossing, and addition of newer versions of the same inbred from other sources. These changes could affect yields of hybrid combinations after several cycles of regeneration. Such variations can be detected by means of phenotypic and genotypic measures.

Plant breeding programmes have traditionally used "phenotypic selection" combining direct measurement in the field, glasshouse or laboratory with statistical analysis, to allow them to search efficiently in large plant populations for progeny that

exhibit desirable traits (Dreher *et al.*, 2003). Morphological variations do not always reflect real genetic variation because of genotype x environment interaction and the largely unknown genetic control of polygenic morphological and agronomic traits (Smith and Smith, 1992).

Development of molecular techniques has provided a tool that can complement these phenotypic selections and make the breeding process even more efficient. These molecular techniques in particular the use of molecular markers have been used to monitor DNA sequences variation in and among the species and create new sources of genetic variations by introducing new favorable traits from landraces and related grass species. A genetic trait is qualified as a genetic marker or DNA marker, if each phenotype can be unambiguously assigned to a set of genotypes at one or more specified loci.

An improvement in markers detection systems and in the techniques used to identify markers linked to useful traits has enabled great advances to be made in recent years. This has been based on complete linkage maps and bulked segregant analysis (BSA). However, alternative methods such as the construction of partial maps and combination of pedigree and marker information have also proved useful in identifying marker traits association.

DNA fingerprinting also known as DNA typing is a DNA-based identification system that relies on genotypic differences among individuals or organism. DNA typing techniques focus on the sequence of the four building blocks (A, T, C and G) of DNA driven by factors like mutation, crossing over, hybridization and environmental changes. Some uses of DNA typing compare the nucleotide sequence of two individuals to see

how similar they are. It is used for any task where minute differences in DNA matter, such as determining the compatibility of tissue types in organ transplants, detecting the presence of a specific micro-organism, tracking desirable genes in plant and animal breeding, establishing paternity, identifying individual remains, and directing captive breeding programs in zoos or farms.

Genetic fingerprinting of maize germplasm is among the techniques used to aid breeders in the identification of breeding lines, populations, pools, and races to the correct heterotic group. The techniques involve the use of Restriction Fragment Length Polymorphism (RFLPs), Random Amplified Polymorphism DNAs (RAPDs), Amplified Fragment Length Polymorphism (AFLPs) and microsatellites or Simple Sequence Repeats (SSRs). This also aids curation of gene bank collections by refining the core subsets formed from field evaluations, and helps to understand the evolution of major tropical maize races better and for legal protection of the parental lines and hybrids.

1.1 Problem statement

At present, inbred lines used at Kenya Agricultural Research Institute (KARI) breeding programs are placed into subsets according to classification on the basis of pedigree (parentage and level of generation) and morphological trait (such as yield and disease information). These have a number of limitations including low polymorphism, low heritability, late expression and vulnerability to environmental influences. There is also insufficient knowledge on the genetic source of the germplasm (parental lines). The lines have been maintained and managed for a long time in the breeding Centre since their acquisition. DNA markers do not have such limitations and therefore can be used to detect variation at DNA level and even distinguishing between closely related genotypes.

1.2 Hypothesis

Genetic variations may occur among maize inbred lines and their populations.

1.3 Justification

Conventional breeding is time consuming and very dependent on environmental conditions. Breeding a new variety takes between eight and twelve years and even then, the release of an improved variety cannot be guaranteed. Hence, breeding requires adopting new technologies that could make this procedure more efficient. This can be achieved through diversity study of parental inbred lines in order to reveal their genetic source.

However, these inbred lines are grouped according to their pedigree and morphological data. Also the lines have maintained for years in the breeding programme and some their genetic source is not known. It is therefore important to refine the major lines by both molecular markers and phenotypic field data.

Therefore, there is need for KARI core inbred lines to be refined by molecular markers or combination of molecular markers and phenotypic field data. Microsatelite markers, or SSRs, have been suggested in other studies, and good correlations have been found between SSR and RFLP diversity and pedigree based measurements for fingerprinting (Viktor *et al*, .2006).

Genetic fingerprinting of maize germplasm can aid the breeders in the placement of breeding lines and populations into the correct heterotic group understand germplasm diversity, understanding the evolution of major tropical maize races better, for legal protection of hybrids and parental lines and improve management strategy for the inbred lines (Pejic *et al*. 1998; Smith *et al*., 1997).

Molecular markers can also be applied in the reduction of the time needed to develop a new variety through markers assisted selection (MAS), increase the overall rate of genetic gain in a breeding cycle, improving traits that were not possible to measure using traditional phenotypic screening and increase access to new genes to provide greater genetic diversity e.g. disease resistance, drought, frost among others (Gupta and Varrshney., 2000).

1.3 General objective

To identify and characterize major maize inbred lines (parents) by morphological traits and Simple Sequence Repeats (SSRs).

1.5 Specific Objectives

1. To characterize ten maize inbred lines using morphological traits e.g. yield, plant and ear height.
2. To characterize ten maize inbred lines using fourteen SSR markers.
3. To analyze the data and classify the accessions into groups based on molecular profiles and morphological traits.

CHAPTER TWO

2.0 Literature Review

2.1 History of maize

Maize is a tall annual crop of the grass family with ten chromosomes. It grows to a height of between 1.5m and 3m. The origins of maize in Kenya provide insights into the varieties cultivated today. The most generally accepted hypothesis is that after being taken from the Americas to Europe in 1494, maize was introduced to the African continent through several routes during the 16th century. Linguistic evidence suggests that the crop penetrated the interior of tropical Africa from the coastal lowland tropics, as part of the Portuguese trade with East Africa (Miracle, 1966). On the East African coast, maize was given many names, including the Swahili name muhindi (the plant of India), and Pemba, the name of the island in the Indian Ocean on which 16th century Portuguese planters cultivated food plants (including maize) to supply their garrison. Although maize was probably known throughout Kenya by the 1880s, up until World War I it seems to have been important as a staple food only along the coastal lowlands in the south-eastern corner of the country. Maize's transition to a major crop in Kenya occurred during World War I, when disease in millet led to famine, and millet seed was consumed rather than planted.

Harrison (1970), Kenya's chief maize breeder during the colonial period when the first plant breeding program was initiated, classified the nation's maize types into four pools. By the turn of the century, the Caribbean flint types of maize dominated the crop area along the East African coast and had spread inland along trade routes, although maize continued to be a minor crop. The rise in the importance of maize occurred after

the introduction of a different gene pool from South Africa, derived from white dent types brought there earlier from North America.

Harrison (1970) reported that through crib selection by European farmers, "Kenya Flat White" emerged as a recognized, reasonably stable population. Two other minor types were also reported, the first, identified as Cuzco, was a high altitude race with strong purple pigmentation that originated from Peru called Githigu in Kikuyu. Cuzco is believed to have been brought by missionaries before World War I, and a variety by the same name is still grown in Kenya. The second minor type, called Local Yellow, was declining in use at the time noted it (Harrison 1970).

The first improved maize variety released in Kenya was the Kitale Synthetic II (an open-pollinated maize variety [OPV] released in 1961), based on inbred lines from the Kenya Flat White complex. Harrison in 1961 made numerous top-crosses using exotic materials and Kitale Synthetic II (Crosses between a selection, line, or clone, and the common pollen parent). The most outstanding was a cross with an unimproved Ecuadorian landrace (Ecuador 574) resulted to Kenya's first varietals hybrid (H611), released in 1964. Since that time, H611 has been the basis of all hybrids developed by the national programs.

2.2 Main growing areas

Since maize is adapted to a whole range of climatical conditions, it is the single most extensively grown crop. Maize needs warm temperatures above 15°c, high rainfall of 1,200mm- 2,500mm, drained light loam soil, and undulating landscape. This facilitates large-scale maize production e.g. Trans-Nzoia and Uasin-Gishu district. Approximately more than 80% of the country's annual maize production is obtained from the mid-altitude and the highland zones.

Maize growing areas in Kenya are broadly classified into four ecological zones based on altitude and annual rainfall. These are (i) the high altitude moist zone, which receive 1200 to 2000 mm rainfall and is at an altitude of 1700 to 2400 meters above sea level. (ii) Mid-altitude moist zone (1200-2000 mm rainfall and an altitude of 1000 to 1700 meters above sea level (masl). (iii) the low-altitude moist zone (less than 1000 masl and 1200-1500 mm rainfall and (iv) the moisture stress zone from 500 to 1800 masl and receives less than 800mm rainfall (see Appendix 2).

Different maize varieties in Kenya are grown as determined by the prevailing conditions of the ecological zones. The chief growing areas are Trans-Nzoia, Nakuru, Bungoma and Uasin-Gishu districts. In South Nyanza, other parts of Rift Valley (Kitale) and Western province, maize is grown alongside other subsistence crops like beans, potatoes and bananas. Good yields are obtained with use of hybrid seeds supplied by Seed Companies and other approved private firms. KARI has developed special maize hybrid such as Katumani composite B, which is adapted to the drier conditions and is grown in Machakos, Kitui, Tana River and Isiolo districts (Appendix 1).

2.3 Uses of maize

Maize is the main staple food of Kenya, averaging over 80% of total cereals namely rice, wheat, millet, and sorghum. A significant portion of the small-scale farmers living in the mid-altitude and highland zones depend on maize production for different purposes. It is mainly consumed in Kenyan households as a thick porridge produced by hand pounding (usually preceded by soaking) or grinding in a hammer mill, followed by boiling or mixed with other foods.

The grains are used in manufacture of corn oil and animal feeds hence it is a vital industrial raw material for the manufacture of starch syrup, alcohol, acetic acid, lactic acid, glucose, paper, rayon, plastic, textile, adhesive, dyes, synthetic rubber, resins, artificial leather, and booth polish (National Institute of Industrial Research, 2004-2007). The stalk leaves, and remains from the maize cobs are used to feed domestic animals especially dairy cattle. The stalks and cobs are also used to provide domestic fuel particularly in the rural areas. They are also used as organic manure.

2.4 Major Maize Constraints

The total land area under maize in Kenya is about 1.5M Ha. 70-80 percent of maize is produced by small-scale farmers with an average on-farm production of 1.5-2.6 tons per ha. This is much lower than the on-station yields which range from 5-8 tons/ha. Inherent challenges to increased maize production are: low productivity-caused by biotic and abiotic constraints; limited utilization of Agricultural technologies and improved seeds, insufficient knowledge on the genetic source of germplasm, low soil fertility, poor infrastructure and marketing policies.

The major biotic causes of stress in maize include the parasitic weed (*Striga*), diseases such as northern corn leaf blight (NCLB), maize streak virus (MSV), common rust, gray leaf spot (GLS), stalk and ear rot, stalk borer, and storage pests such as common weevils and large grain borers.

2.4.1 Maize streak virus

The disease occurs in all African countries south of Sahara India ocean islands and causes severe yield losses in the lowland humid and savanna areas as well as in the mid-altitude ecological systems (1000-1800m) (Anonymous, 1982a.b). The virus is spread by a small leafhopper, *Cicadulina mbila*. The symptoms occur as longitudinal light-yellow streaks along the leaf veins, photosynthesis is impaired, resulting to stunt growth and yield reduction. It causes up to 100% yield loss when epidemics occur on susceptible genotypes mainly due to loss of photosynthetic chlorophyll. Control of MSV is complicated by the fact that it exists in many forms, varying from region to region, (Rybicky, 1994).

2.4.2 Gray Leaf Spot

Gray leaf spot caused by *Cercospora zea-maydis* is a highly weather-dependent disease. The pathogen requires long periods of high relative humidity and free moisture (dew) on the leaves for infection to occur. The lower leaves of the corn plant are most often the sites of initial infections. When conditions are favorable for disease development, conidia are produced in lesions on the lower leaves and serve as inoculums for the upper leaves. If conditions are not favorable for disease, the fungus can remain "dormant" during the dry part of summer and then become active when favorable

conditions return. Under periods of prolonged favorable conditions, severe blighting can occur.

Symptoms include; necrotic lesions, increased lodging and premature death of the crops. The lesions make the entire leaf area to blight thus reducing the photosynthetic area causing poor grain filling reducing the yield potential of susceptible cultivars by 50% and 88% in high disease pressure (Raymundo and Hooker, 1981).

2.4.3 Common rust

Common rust is also found worldwide and has been reported to cause economic losses on some 7.8 million hectares or 34% of the maize in subtropical through –highland maize ecologies. Head smut has been reported in the USA, Mexico, Austria, New Zealand ,Africa, Southern Europe and parts of Russia and causes yield and quality reduction (Njuguna, 1999).

2.4.4 Northern Corn leaf blight (Turcicum blight)

Outbreak of turcicum blight in Kenya was first reported in 1999 (Gebrekidan *et al*,. 1992). Highland blight is one of the most economically important diseases (Njuguna *et al*, .1990). Reports indicate that the commercial varieties and super elite breeder's materials are vulnerable to the highland blight (KARI Maize Data Base, 1994). Turcicum blight is considered a serious disease where climatic conditions are cool with high relative humidity. Yield losses have approached 50% when the disease is severe at 2-3 weeks after pollination (Shurtleff, 1980).

Host plant resistance is the cheapest and most effective way to control leaf blight disease because chemicals treatments are expensive, often ineffective and sanitation

practices in crops such as maize are difficult to apply. Performance of the new hybrids in presence of *turcicum* blight has also been improved from a score 4.0 to a score of 1.5 (4.0 susceptible and 1.5 resistance) (Jane *et al.*, 2005).

Northern maize leaf blight is favoured by mild temperature and high humidity. Heavy dews, cool temperatures, and frequent rains create good sustained environmental conditions for disease development. Levy (1991) showed that isolates from different areas were different in parasitic fitness as was indicated by infection efficiency, sporulation and lesion size, while isolates of same location showed less variation. Inoculum in previous crop has been found to be critical in epidemic build up for subsequent cropping, especially in non tillage systems.

2.4.5 Head smut

Head smut in maize and sorghum is caused by *Sporisorium reilianum*. In maize both tassel (male inflorescence) and cobs (female inflorescence) may be partially or completely smutted. Grain yield reduction from smut diseases alone is estimated to be 5% (Njuguna *et al.*, 1990).

2.4.6 Pests

Stalk borer and storage pests such as weevils and large grain borers are the major pests. Stem borers constitute one of the major factors limiting efficient maize production in the country. The two major species of stem borers of maize in Kenya are *Chilo partellus* and *Busseola fusca*. *Chilo partellus* is prevalent in the dry low attitude and humid agro-ecological areas whereas *Busseola fusca* is commonly found in the wetter, cooler and highland zones. *Chilo partellus* comprises 90% of all borer species infesting maize in

Kenya, causing yield losses of about 18% to 40 % Stem borers consume 400,000 Tonnes of maize, inflicting an average 15% annual yield loss. Such large losses are estimated at US$72 million per year in Kenya alone, particularly the pink stem borer *(Chilo partellus)* (Bigirwa et al., 2001)

2.4.7 Striga

Striga hermonthica (Del) Benth. and *Striga asiatica* (l.) Kuntze are weeds that infest million of hectares of land under cereals in sub Saharan Africa, threatening food security. The problem of Striga intensifying across regions in sub-Saharan Africa is because of deteriorating soil fertility, shortening of the fallow period, expansion of production into marginal lands with little nutrient input and an increasing trend towards continuous cultivation of one crop in place of traditional rotation and inter-cropping systems. Striga infests 400,000 hectares of farmland each year in Kenya alone. The effects are likely to be long lasting as Striga plants produce millions of tiny seeds that can stay viable in the soil for many years (Ejeta and Buther, 1993).

2.5 Maize breeding and Improvement

Plant breeding is a combination of principles and methods of changing the genetic constitution of a plant to make it more suitable for human need. It combines of science and art, ability of a breeder to identify differences in the traits of economic importance among plants and improving these traits with available scientific knowledge. Conventional plant breeding involves crossing of quality plant possessing the best and desirable traits (e.g., high yield or disease resistance) that are required by farmers.

Since almost all the arable land is under cultivation in Kenya, future increases in maize production will heavily depend on yield improvement rather than expansion in arable area (Karanja and Oketch, 1992). The most effective and economic means of controlling the major constraints is resistance breeding. The core breeding strategy that has resulted in major genetic improvement of maize grain yield and other desirable agronomic traits in Kenya, in the recent years was the use of pedigree breeding, combined with extensive multi-location testing which was designed to asses the phenotypic performance of new genotypes across a large sample of environment.

In the past, breeding for resistance in maize disease in Kenya was done without sufficient field data resulting in a scenario where inbred lines and hybrids were produced without any field and green house disease challenges. This ended up producing hybrids of high disease risk which has contributed to low yields (Ayub-Takem and Chheda, 1982). By then over 90% of the crop diseases were reported in research and extension annual reports but very little was known about disease incidence and severity, pathogen distribution, epidemiological, yield losses and physiological specialization which were vital basic data in plant breeding. The approach used to identify desirable germplasm involves the exchange of germplasm used in conventional breeding programme among centers of the Kenya Agricultural Research Institute (KARI), seed companies CIMMYT among others. Thus strengthening resistance breeding against the major pests and diseases is a requisite and this can be done mainly through: (i) Identification of genotypes with inherent resistance to maize streak disease, gray leaf spot, northern corn leaf blight such as Ecuador 573 and Kitale synthetic; (ii) use of elite inbred lines and populations in the formation of novel varieties and (iii) the release of hybrids, open pollinated varieties

(OPVs) and inbred lines resistant to foliar diseases. This will contribute in alleviating poverty in these areas.

CIMMYT maize lines (CMLs) are carefully selected inbred lines from CIMMYT (Zimbabwe-Harare) with good combining ability and significant number of value adding traits such as drought tolerance, nitrogen use efficiency, acid soil tolerance to diseases, insects and parasitic weeds. They are successful in one or several maize major environment.

EM11-133 and EM 12-210 which are the two lines that were developed in the early 60's from Embu composites Kenya mid-altitude lines of desirable agronomic character, but susceptible to major maize diseases. Both lines are of good combining ability, high yield and been used in the development of major hybrids in Kenya e.g. H513 and H511

OSU 23i originated from Ohio State University U.S.A. It is of high resistance to MSV with a mean score of < 2.0 (light streaking on older leaves) but of poor combining ability and yield. It has been integrated in maize breeding programmes in the development of lines of high adaptability in Kenya but resistance to MSV and other major maize constraints. For this reason, it has resulted to the release of MSV resistance hybrids such as Muguga 1 and MU99023.

The goal of maize breeding program at KARI-Muguga is to develop maize varieties for mid-altitude areas of Kenya, which will contribute positively to on-farm productivity from the current 1.5-2.0 metric tons per hectare to above 4.5-6.0 tons per hectare thereby increasing rural household incomes. This is accomplished by developing maize varieties with a high genetic potential, resistant to foliar diseases and pests, and

other value added traits such as a high nutritional value and foliage. The produced hybrids must also fit in early maturity groups of 100-120 days, 120-140 days and 140-160 days.

Other places inbred lines and hybrids are screened other than KARI-Muguga are KARI-Katumani for drought resistance and earliness, KARI-Embu for stem borer resistance and KARI-Kakamega for gray leaf spot, striga, common rust and northern leaf blight resistance. The selected lines based on independent culling selection are used to develop single crosses, three-way crosses, top crosses and double-cross hybrids. These crosses are evaluated through the maize testing system in Muguga, coast, eastern and in western Kenya, depending on the stage of testing. Data taken during variety evaluation include relevant disease scores, plant and ear height, maturity and grain yield among others.

KARI Kakamega has released hybrids such as: KH633A, KH634A, KM20077, KM20084 and KM20090, which have been identified as high yielding and resistant to gray leaf spot, and northern leaf blight. Grain yields are 9.3 T ha^{-1} for KM20077, 9.7 T ha^{-1} for KM20084 and 10.7 T ha^{-1} for KM20090 compared with the commercial checks: KN633A (7.2 T ha^{-1}), KN634A (7.7 T ha^{-1}) and PH 3253 (5.2 T ha^{-1}).

Other inbred lines produced by KARI Muguga include; MU007, MU016, MU002, MU015, DC17, DC31, DC96 and DC complex population which are significantly better than the commercial checks yield mean of 10.78 T ha^{-1}. Selection is also done for adaptability, yield and resistance to MSV and common rust. Eighty five percent of the lines of MU007 have shown a highly resistant score (< 1.5) in MU016,

89%; MU002 and MU015 populations both had 14% of lines with high resistance to MSV and 23% with moderate resistance.

Thirty-seven lines selected from two populations, MU15 and MU002, were used in population improvement. Crosses were evaluated for performance in Kakamega and Bukura to identify the best-performing three-way cross hybrids. Twenty hybrids have been identified as best performers in terms of grain yield and resistance to MSV (Danson, *at el.*, 2005).

Three inbred lines (Diplo pool, LE pool and TZL pool from Tanzania) classified as resistance to Striga were crossed with adapted but susceptible inbred lines, CML-204, CML-444, CML-312, CML442, CML-373, and CML-395 from CIMMYT, and MUG-1, MUG-2, MUG-3 and MUG-4 from KARI which were developed from EM11-133, EM12-210, OSU23i and CML202. The hybrids were evaluated for two seasons at KARI Muguga for *Striga asiatica* and at Kibos and Alupe for *Striga hermonthica*. Diplo pool and LE pool were better sources of resistance to *Striga hermonthica* while LE pool and TZL pools were better sources of resistant to S. *asiatica*. From the crosses, a new maize hybrid variety was developed which shows high resistance to Striga. The new hybrid could raise crop production in Kenya by as much as 200,000 tons.

CIMMYT has also developed quality protein maize (QPM) through conventional breeding methods using opaque-2 mutants that have double the level of lysine and tryptophan. QPM has 90% of the nutritive value of milk protein but looks and tastes like normal maize and has similar yield and agronomic performance. Lysine also aid in assimilation of zinc and iron from maize grain.

The divergent-convergent breeding approach has enabled the KARI maize-breeding program to identify desirable inbred, populations, synthetics and hybrids in shorter time than it could have taken if individual KARI centers had been working independently. The approach has also encouraged sharing of information and germplasm among the maize breeders.

2.6 DNA Fingerprinting

DNA fingerprinting, also called DNA profiling or DNA typing, makes use of segments of DNA that do not code for protein products, but do exhibit variability caused by mutation in the nucleotide base sequences from individual to individual. In some instances the segment of DNA being investigated contains varying numbers of repeated letters from one individual to another, like a molecular stutter. When these segments of DNA are cut using restriction enzymes, DNA fragments of various lengths are produced. If the DNA of an individual has mutations within restriction sites the DNA will not be cut at those sites and that individual DNA fragments will be different in length from another DNA fragments produced by the same restriction enzymes. Examples of DNA fingerprinting are to;

(i) To build molecular maps of plant and other genomes;

(ii) To analyze genetic variation within and between specific populations, as in DNA fingerprinting;

(iii)To assist in otherwise conventional plant breeding

The technique has now spread to plant biotechnology.

DNA fingerprinting was first developed in England in 1985 and takes advantage of the fact that with the exception of identical twins, the genetic material of each person or individual is unique. When the National Research Council said in a 1992 study that DNA

testing was a reliable method to identify criminal suspects, the technology rapidly entered the mainstream court system Today, it is hard to pick up a daily paper and not find an article reporting on the use of DNA testing in a civil or criminal court case.

2.7 Molecular Markers

Tropical maize germplasm have not been fully classified into well-defined heterotic groups and pedigree information is not available for some of the inbred lines, synthetics and populations used in breeding programs. These have led to difficulty in identifying favorable alleles in the genotype to generations through repeated cycles of selection and identifying individual plants in large segregating populations that carry the desirable alleles. The study of genetically diversity could assist breeders to move efficiently to choose genetic diverse parents for breeding programs and systematically introgress traits from new germplasm.

Molecular markers information helps to monitor the level of genetic diversity in breeding materials, as well as the purity of inbred lines. Morphological and isoenzymes markers although cheap and easy to use, have a number of limitations, which include low polymorphism, low heritability, late expression and vulnerability to environmental influences. DNA markers on the other hand do not have such limitation and they can be used to detect variation at the DNA level this has proven to be an effective tools for distinguishing between closely related genotypes (Helentjaris & Burr, 1989, Doeblay et al, 1984; Eyre-Walker et al, 1998; Hilkton and Gaut 1998). Molecular markers can also be used to pyramid genes for traits that are not possible to measure using traditional phenotypic screening e.g., frost tolerance, pre-harvest sprouting, late maturity, drought

tolerance, tolerance to exotic pests e.g. stem borers, diseases among them MSV, Turcicum blight, common rust and gray leaf spot. Molecular markers based on DNA variations are independent of environmental effects, can be assayed easily at any stage of the plants development and are free from pleotropic or epistatic effects (Crouch and Tenkouano, 1999).

2.7.1 Types of molecular markers

(i) Different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Length Polymorphisms (RAPDs), Simple Sequence Repearts (SSRs) or miscrosatellites, Amplified Fragment Length Polymorphisms (AFLPs), Single Nucleotide Polymorphisms (SNP), Sequence Tagged Site (STS), minisatelites and others have been developed and applied in crop improvement (Marsan *et al.*, 1998).

2.7.2 Restriction Fragment Length Polymorphism (RFLP)

RFLP's been the first to be developed (some 15 years ago) and have been widely and successfully used to construct linkage maps of various species, including maize and wheat. RFLP's markers are highly reproducible, have high genomic abundance, are randomly distributed throughout the genome and profiles can be interpreted in terms of loci and alleles. Although their filters can be washed and reproduced several times, they require large quantities of purified high molecular weight DNA (5-10μg), are laborious and technically demanding. High cost is incurred in case suitable probes are unavailable; they are not PCR based and amendable to automation because is dependant to the presence of the recognition sites (Messmer *et al.*, 1993; Benchimol *et al.*, 2000).

2.7.3 Random Amplified Polymorphic DNAs (RAPDs)

With the development of Polymerase Chain Reaction (PCR) technology several markers types emerged. The first of those were RAPD markers which quickly gained popularity over RFLPs due to the simplicity and decreased costs of the assay. However, most researchers now realize the weaknesses of RAPDs and use them with much less frequently. Some of these weaknesses include; the requirement of purified and high molecular weight DNA is required. Precautions are needed to avoid contamination of DNA; highly standardized experimental procedures are needed because of sensitivity to reaction conditions. The process is also limited because profiles cannot be interpreted in terms of loci and alleles, dominance of alleles, low reproducibility and similar sized fragments may not be homologous (Dos Santos *et al*., 1994; Thormann *et al*., 1994).

2.7.4 Amplified fragment length polymorphisms (AFLPs)

AFLPs takes advantage of the PCR technique to selectively amplify DNA fragments previously digested with one or two restriction enzymes. Playing with the number of selective bases of the primers and considering the number of amplification products per primer pair, this approach is certainly powerful in terms of polymorphisms identified per reaction. AFLPs on the other hand are easy to use, have lower initial cost, are PCR-based, require little amounts of DNA (0.5-1.0ug), and highly reproducible. Scoring AFLP data is simple and easy because a large number of polymorphic bands in a single lane rather than high level of polymorphism at each locus can be detected. (Gerber *et al*., 2000)

2.7.5 SSR (Microsatellite) markers

SSRs is a marker system, which has recently been developed as anonymous RAPD-like approach that access variation in numerous microsatellite regions which are, dispersed throughout the various genomes (particularly the nuclear genome). Microsatellites are composed of tandem repeat of two to six nucleotide DNA core sequences such as (AT) spread throughout the eukaryote's genomes. Weber and May (1989) demonstrated their high level of polymorphism in which is due to variations in the number of tandemrepeats, abundance and even distribution across the genome. They are characterized by mono-, di-, tri-, tetra-, or pentanucleotides repeat types.

Di and trinucleotides repeat types are targeted because they are characteristic of the nuclear genome (Vos *et al*, 1995) and their occurence in chloroplast as well as in mitochondria genome (Soranzo *et al*, 1999); which increased their application in maize (Chin *et al*, 1996). The application of SSR techniques to plants depends on the availability of suitable microsatellite markers, which have been developed for species such as soyabean (Rongwen *et al*, 1995), rice (Zhao and Kochert, 1993), maize (Taramino and Tingey, 1996), the common bean (Yuan *et al*, 2000) and barley (Saghai *et al*, 1994).

SSR marker system is advantageous since it requires low quantities of template DNA (10-100ng per reaction) and markers are randomly distributed throughout the genome. The resulting band profiles can be interpreted in terms of loci and alleles and allele sizes can be determined with an accuracy of one base pair, allowing accurate comparison across different gels. A high number of public SSR primer pairs are available in maize database (http://nucleus.agron.missouri.edu/cgi-bin/ssr-bin.pl; verified 15 July

2004) and the heterozygosty of SSRs is seven to ten times higher than that of RFLPs (Wu and Tanskley, 1993). Since microsatellites are co-dominant markers, the level of polymorphisms in plant species studied has been greater than that found with other markers. They may also be used across species and genus boundaries.

These molecular markers have technical differences in terms of cost, speed, amount of DNA needed, technical labour, degree of polymorphism, precision of genetic distance estimates and the statistical power of tests and facilities available (Viktor *et al.*, 2006, Table 1).

Table 1. Comparison of the most common used marker systems in cereals

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	high	high	moderate	moderate	high
PCR-based	no	yes	yes	yes	yes
Number of polymorph loci analyzed	1.0-3.0	1.5-5.0	20-100	1.0-3.0	1.0
Easy of use	not easy	easy	easy	easy	easy
Amenable to automation	low	moderate	moderate	high	high
Reproducibility	high	unreliable	high	high	high
Development cost	low	low	moderate	high	high
Cost per analysis	high	low	moderate	low	low

Source: Viktor *et al.*, 2006

The information in table 1 suggests that RFLP, SSR and AFLP markers are the most effective in detecting polymorphism. However, given the large amount of DNA required for RFLP detection and the difficulties in automating RFLP analysis, AFLPs and SSRs are the most popular markers in maize. Other applications of molecular markers include:

(ii) Assessment of genetic variability and characterization of germplasm;

(iii) Identification and fingerprinting of genotype;

(iv) Estimation of genetic distance between population, inbreds and breeding material;

(v) Detection of monogenic and qualitative trait loci (QTLs);

(vi) Marker-assisted selection (MAS);

(vii) Identification of sequences of useful candidate genes, etc

2.7.5.1 Applications of Simple Sequence Repeats markers in maize breeding

It is vital for plant breeding programmes to have sufficient diversity available to allow for the production of new varieties that are aimed towards the improvement of crop productivity and able to withstand damage from biotic and abiotic factors. SSR markers have been used as a tool to identify major genes, or to introduce new characters in elite germpalsm.

Microsatelite markers can ensure the presence of multiple genes, hence its application in selecting stable and durable resistance maize varieties for the major constraints. QTLs for turcicum blight and gray leaf spot disease resistance have been mapped on chromosome 2 and 8 in CML 202, chromosome 10 for gray leaf spot and common rust, and chromosome 1 for gray leaf spot and maize streak virus (Danson *et al.*, 2006).

The use of SSRs in maize allows plant selection at the juvenile stage from an early generation. Unfavorable alleles are eliminated or greatly reduced during the early stages of plant development through marker assisted selection (MAS), focusing the selection in the field on reduced numbers of mature plants (Viktor *et al*, .2006).

SSRs have also been used in genetic identification of favorable alleles, estimation of genome size. Population genetics, DNA fingerprinting for legal protection of hybrids and parental lines (Senior *et al,* 1988).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Planting materials

A total of ten maize inbred lines collected from KARI-Muguga were used in this study

(Table 2).

Table2: Characteristics of maize lines used in the study (Source: CIMMYT 2004)

Inbred line	*Germplasm resource*	*Country of Origin*	*Pedigree*	*Plant heigt (cm)*	*Ear heig ht (cm)*	*Days to tasseling (DTT)*	*Days to silking (DTS)*	*Grain Color*	*Grain Texture*	*Stress tolerance &resistan ce*
EM11-133	KARI-Muguga	Kenya-Embu	Not available	158	105	107	114	YW	F	none
EM12-210	KARI-Muguga	Kenya-Embu	Not available	166	88	88	97	W	F	none
OSU23i	KARI-Muguga	Ohio State University	Not available	130	71	68	71	W	F	MSV
CML202	KARI-Muguga	CIMMYT(Harare)	ZSR923S4BULK-5-1-BB	140	80	114	116	W	SD	MSV, E. turcicum, P. sorghi
CML204	KARI-Muguga	CIMMYT(Harare)	[7794]-SELF-4-1-S9-1-4-7-4-5-BB	160	100	102	107	W	D	MSV, E. turcicum, P. sorghi
CML206	KARI-Muguga	CIMMYT(Harare)	[EV7992#/EVPO44SRBC3]#BF37SR-2-3SR-2-4-3-BB	120	60	160	124	W	SD	MSV, E. turcicum, P. sorghi
CML312	KARI-Muguga	CIMMYT(Harare)	S89500F2-2-2-1-1-B*5	201	75	102	114	W	SF	GLS, E turcicum
CML395	KARI-Muguga	CIMMYT(Harare)	90323(B)-1-B-1-B*4	190	67	81	115	W	SF	MSV
CML442	KARI-Muguga	CIMMYT(Harare)	[M37W/ZM607#BF37SR-2-3SR-6-2-X]-8-2-X-1-BBB	146	74	75	104	W	D	Drought, Low N
CML444	KARI-Muguga	CIMMYT(Harare)	P43C9-1-1-1-1-1-BBBB	144	81	82	93	W	SD	Drought, Low N

ZM=Zimbabwe maize; B=selfed and bulked; -1,-2,-3…=ear to row; # = sibbing; SR =streak resistance; F=full sib; EV=Experimental variety; BC=back cross; F=Flint; SD= Semi-dent; D= Dent; SF= Semi-flint; YW= Yellowish white; W= White

3.2 Planting

The experiment was conducted at KARI- Muguga south research centre which is in Kiambu District, Central Province of Kenya, located at an altitude of 2095m, latitude 36° 34-36° 39`S and longitude 1° 11`-14`E. The mean annual rainfall is 946 mm. The soil types are Nitisols according to FAO/UNESCO classification and Alfisols according to USDA classification (www.kari.org). The experiment was conducted from April 2006 to December 2006. Seeds were sown on 07th April 2006. The maize inbred lines (Table 2) were planted at in a one row plot at a spacing of 75cm between rows and 30cm within rows this was to allow for full plant growth for determination of morphological trait data using randomized complete block design (RCBD). Each row had 25 plants, which constituted 44,444 plants per hectare recommended for the testing site.

This experiment was planted off-season and required irrigation throughout the trial period. Diammonium phosphate (DAP) was applied during planting at a rate of 3g per hill (80kg P_2O_5 and 31 kg N/ha) while CAN (Calcium Ammonium Nitrate) was applied as a top dress 6 weeks later at rate of 2g per hill (80kg N/ha). Carbofuran (2-3, -dihydro-2, 2-dimethyl-7-benzofuranol methylcarbamate) was applied at a rate of 3g per hill to control cutworms. Bulldock 0.05 GR (Beta-cyfluhtrin) was used to control stalk borers and cutworms. The field was kept free of weeds by hand weeding and irrigated throughout the growing season.

3.2 Morphological traits

Ten plants were selected at random from each inbred line and evaluated for morphological traits. Data were recorded for several morphological traits as follows:

(i) *Days to silking (DTS)*: Number of days between emergence and when 50% of plants in each plot were having 1cm or more of exposed silk.

(ii) ***Days to pollen shed (DTT)***: Number of days between emergence and when 50% of the plants in each plot were shedding pollen.

(iii) ***Ear height (EHT-cm):*** The height from the ground level to the node bearing the upper most ears recorded as a mean of ten randomly selected plants.

(iv) ***Plant height (PHT-cm):*** The height from the node of the flag leaf to the soil level recorded as a mean of ten randomly selected plants.

(v) ***Grain Yield (GY-t/ha):*** The fresh weight of all harvested cobs for each plot was taken and recorded. Grain moisture content was determined from a seed sample from ten randomly selected cobs. The weight of the harvested cobs was then adjusted to 12.5 % moisture content and while assuming 80% shelling percentage, yield in Tonnes per hectare was calculated as follows; using the equation below

Yield = [Field Weight/ Plot size] * [(100- MC %) / 87.5] * 0.8 * 10.

(vi) ***Kernel weight (200SWT-grams):*** A sample of 200 kernels from freshly harvested cobs per harvested plot was weighed and the weight adjusted to 12.5 % moisture content.

(vii) ***Ear length (EL-cm):*** This was determined for five randomly de-husked cobs per plot, which were measured from the collar (base) of the ear to the tip where grain filling ends.

(viii) *Ear Aspect (EA):* This is the relative measure of the overall appeal of harvested ears and was assessed visually on a scale of 1-5, 1 for excellent appearance and 5 for poorest appearance. Extent of grain filling and alignment of kernels on the cob were some of the factors considered when assessing ear aspect.

(ix) ***Root and Stalk lodging (RL and SL):*** The total number of plants in each plots which either lodged at the roots (RL) or stem (SL) was recorded and converted as a percentage.

(x) ***Bare Tips (BT):*** All the cobs with exposed tips were counted and recorded as a percentage of total number of plants assayed.

(xi) ***Ear placement (EP):*** This was calculated as the ratio between plant height and ear height.

(xii) ***Ear prolificacy (Eprol):*** This was determined as the mean number of ears per plant.

Other morphological data collected include; angle between blade and stem at the beginning of anthesis (small, medium or large); anthocyanin coloration of the sheath when the grains were watery ripe; glumes at the base and tassels at halfway anthesis (very weak, weak, medium, strong, or very strong); width of the leaf at the medium milk grain stage; intensity coloration of silk at halfway anthesis, among others.

3.4 Molecular characterization

3.4.1 Sampling and lyophilization of leaves

Two young leaves were sampled randomly from five week old maize plants. The leaves were cut and folded (Fig 1a and b) and put separately in perforated bags before being transported to the laboratory in ice-cubes. The leaves were stored at –80°C until ready for lyophilization. The lyophilizer was set down to a temperature (≤ -60°C) and pulled to a good vacuum (≤10 microns Hg) before loading the samples. The vacuum was set at 100 micron Hg and a condenser temperature of –60°C.The samples were freeze-dried for 72 hours and stored in a sealed plastic bag at room temperature until ready for DNA extraction.

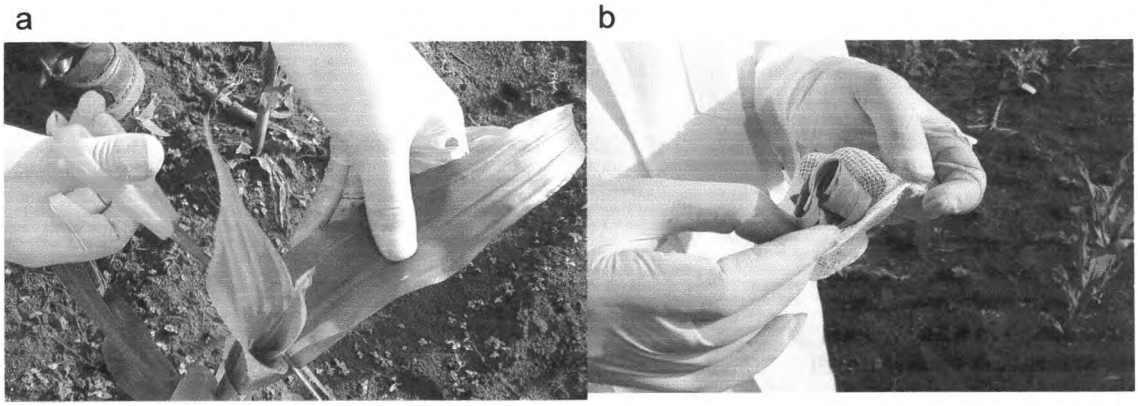


Fig 1: (a) Cutting maize leaves with a sterile pair of scissors (b) Wrapping the folded leaf into a net bag after removing the mid-rib.

3.4.2 DNA extraction

3.4.2.1 Grinding

The lyophilized leaf samples were chopped into half-inch segments with a sterilized pair of scissors and placed in pre-chilled mortar. Liquid nitrogen was added to quickly freeze-dry the samples before grinding to fine powder with pestle (Fig. 2). The

ground material was placed in a 15ml polypropylene centrifuge tube and stored at - 20^0C until used as a source of DNA.

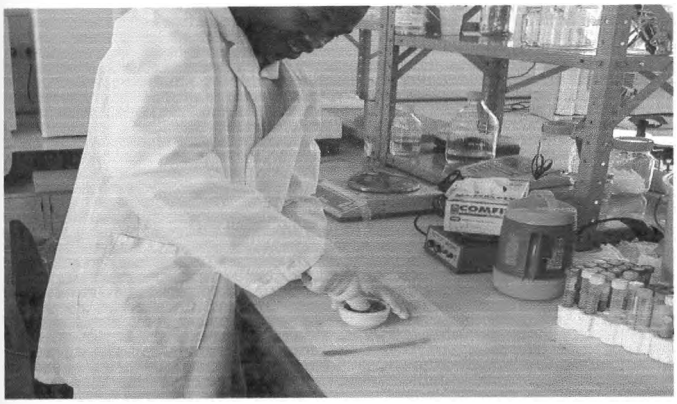


Fig 2: Grinding of leaves

3.4.2.2 DNA Extraction using CTAB method

Genomic DNA was extracted using Cetyl Trimethly Ammonium Bromide (CTAB) method described by Shaghai and Maroof 1984 and modified by Hoisington *et al.*, 1994. 0.3g of the ground material was weighed into new 15ml polypylene centrifuge tube. Immediately, 9ml of pre-warmed (65^0C) CTAB extraction buffer (700 mM Sodium Chloride, 100mM Tri HCL PH 7.5, 50mM EDTA PH 8.0, 140 mM β-mercaptoethanol and 1% CTAB) was added and tubes incubated for one hour with a continuous gentle mixing in oven at 65^oC to lyse the cells. The tubes were removed from the oven and cooled briefly for 4-5 minutes. To the homogenate, 4.5ml of chloroform/octanol mixture (24:1) was added and the tubes capped tightly, mixed by gentle rocking with medium circular motion using a shaker for 15 minutes. This formed a thick emulsion between the DNA phase and the chloroform/octanol (24:1) phase. The homogenate was centrifuged for 10 minutes at 3500 rpm at room temperature (22 ± 2^0C).

The top aqueous layer was pipetted out and transferred to a new 15ml polypylene centrifuge tube. To the supernatant, 4.5ml of chloroform/octanol (24:1) mixture was

added, rocked gently for 10 minutes on a shaker and centrifuged at 3500 rpm for 10 minutes at room temperature

The top aqueous layer was again pipetted out and transferred to a new 15ml polypylene centrifuge tube and 20μl of 10mg/ml pre-boiled RNase A added, mixed by gentle inversion and incubated for 30 minutes at room temperature. DNA was precipitated by adding 6ml of ice-cold absolute ethanol and mixed gently. The precipitated DNA was hooked out with a sterile glass Pasteur pipette hook and placed in a 1.5ml micro-centrifuge tube containing 500μl wash 1 buffer (containing of 76% ethanol and 0.2M sodium acetate) and washed for 5 minutes. Wash 1 was poured out while holding the DNA on the glass hook, and washed with 500 μl of wash 2 (consisting of 76% ethanol and 10mM ammonium acetate) and washed for 5minutes. This second wash was poured out and the DNA air-dried for 30 minutes. The DNA was re-suspended in 300μl of 0.1X TE (10mM Tris, 1mM EDTA- pH 8.0) at 37°C for 1 hour while mixing every 15 minutes by gentle inversion to help speed up the process.

3.3.3 DNA Quality and Quantity

The quality of genomic DNA obtained was assessed by agarose gel electrophoresis. A 0.8% agarose gel was prepared by weighing 0.8g (Sigma type I: Low EEO) of agarose in a 250ml beaker containing 100ml of 1X TAE buffer (160ml 2.5M Tri HCL, 0.23ml glacial acetate acid, 0.4ml 0.5M EDTA and 39.37ml double distilled water) and swirled to mix. The mixture was boiled in a microwave oven and allowed to cool to about 45°c.The molten gel was stained by adding 2μl of ethidium bromide (10mg/ml ultra PURE from GIBCO BRL) solution was added to the molten gel before pouring into a medium gel mold with two 14 well combs. The gel was allowed to solidify evident

when the gel turned cloudy and hard before placing in a gel electrophoresis tank containing 1XTAE buffer. The gel was completely submerged prior to removing the combs.

Each DNA sample was mixed with 1 X gel loading dye (65% (W/V)) sucrose, 10mM Tris-HCl (PH 7.5), 10mM EDTA, and 0.3% (W/V) bromophenol blue) at a ratio of 5 μl of DNA to 2μl of the dye, loaded into the wells, before electrodes of the gel tank were hooked up, and power (Sigma PS 2000-2 power supply) turned on to 80 volts. After running for about one hour, the gel was removed, visualized on a UV transilluminator box and photographed using Polaroid camera and film. From the photographs the quality of the DNA was checked and the sheared DNA samples were re-extracted. Traces of RNA were removed by re-incubating the DNA with 5μl of RNAse A (10mg/ml) for one hour at 37^{0}C (Fig .3).

The concentration of the genomic DNA was determined on the basis of optical density readings. From each stock DNA sample, a 15μl aliquot diluted in 735μl of 1XTE buffer was prepared, and its optical density (ODs) determined at wavelength s 260 *nm* and 280 *nm* on a spectrophotometer (Ultrospec 2000, Pharmacia) . The concentration of the DNA in samples was determined as follows: One OD unit approximately 50μg double stranded DNA per ml, and 15μl of sample in 750μl cuvette in a dilution of 50 times using the equation below.

DNA concentration (μg/ μl) = OD260 X 50 (dilution factor) X 50 μg/ml/1000

3.4 Polymerase chain reaction (PCR)

3.4.1 DNA dilution

After calculating the concentrations in µg/µl, the stock DNA solution was diluted to 30*ng/ µl* working solution for polymerase chain reaction (PCR) using the formula;

$M_1V_1=M_2V2$

(300*ng/ µl*) (V1)=30*ng/ µl* (100ul)

V1= (30*ng µl* /) (100 *µl*)/(300*ng/ µl*)

V1=10 *µl*

Where M1 is the stock DNA concentration (300*ng/ µl*)

V1 is the volume of the stock to be diluted (10 *µl*)

M2 is the concentration of working solution (30*ng/ µl*)

V2 is the volume of working solution to be prepared (100*µl*)

The working solution was stored at 4^0C.

3.4.2 Amplification (SSR analysis)

A total of 14 SSR primers were selected from previous studies (Warburton *et al.*, 2002; Matsuoka *et al.*, 2002) and from the public maize Data Base (DB) (http://www.agron.missouri.edu/ssr_p robes/ssr.htm) based on their high polymorphism information content and chromosome location (at least 2 SSRs per chromosome) (Table 3). PCR was performed in 20 *µl* reaction mixes consisting of 30ng template DNA, 1.5mM $MgCl_2$, 0.8mM dNTP mix, 0.5 *µM* SSR primers (forward and reverse), 0.125U Taq polymerase (Roche) and 1x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$) in a 1.5ml micro-centrifuge tube on ice. In a 20 *µl* PCR reaction volume, 18 *µl* of master mix was mixed with 2 *µl* of 30*ng* DNA. The reaction mixture was

vortexed gently and run in a GeneAmp cycler (GeneAmp PCR system 2700 from Applied Biosystems) with the following PCR program: Initial denaturation at 95^0C for 5 minutes, followed by 30 cycles of 94^0C for 30 seconds, 56^0C for 45 seconds and 72^0C for 30 seconds. This was followed by one final extension cycle at 72^0c for 10 minutes, and an indefinite hold at 4^0c. The SSR amplification products were resolved on 2% agarose gel in 1X TAE buffer. Gels were run in a medium format horizontal gel system at 100V for 45 minutes and were photographed under UV light (Fig.8). This was repeated twice for accuracy.

Table 3. SSR markers used to study differences in the maize inbred lines. (source:http://www.maizegdb.org)

Marker name	Repeat*	Bin**	Annealing temperature	Sequence (Forward/Reverse Primer)(5 -3)
Umc1122	Tri	1.06	61	CACAACTCCATCAGAGGACAGAGA// CTGCTACGACATACGCAAGGC
Phi339017	Tri	1.03	53	ACTGCTGTTGGGGTAGGG // GCAGCTTGAGCAGGAAGC
Phi101049	Tetra	2.09	49	CCGGGAACTTGTTCATCG // CCACGTCCATGATCACACC
Phi127	Tetra	2.08	55	ATATGCATTGCCTGGAACTGGAAGGA // AATTCAAACACGCCTCCCGAGTGT
Phi053	Tetra	3.05	59	CTGCCTCTCAGATTCAGAGATTGAC// AACCCAACGTACTCCGGCAG
Phi029	Comp.	3.04	55	TTGTCTTTCTTCCTCCACAAGCAGCGAA// ATTTCCAGTTGCCACCGACGAAGAACTT
Phi331888	Tri	5.04	49	TTGCGCAAGTTTGTAGCTG // ACTGAACCGCATGCCAAC
Phi423796	Penta.	6.02	61	CACTACTCGATCTGAACCACCA// CGCTCTGTGAATTTGCTAGCTC
Phi328175	Tri	7.04	53	GGGAAGTGCTCCTTGCAG // CGGTAGGTGAACGCGGTA
Phi034	Tri	7.02	61	TAGCGACAGGATGGCCTCTTCT// GGGGAGCACGCCTTCGTTCT
Phi100175	Tetra	8.06	47	TATCTGACGAATCCCATTCCC// GTACGTAACGGACGGACGG
Phi233376	Tri	8.03	53	CCGGCAGTCGATTACTCC// CGAGACCAAGAGAACCCTCA
Phi059	Tri	10.02	61	AAGCTAATTAAGGCCGGTCATCCC// TCCGTGTACTCGGCGGACTC
phi041	Tetra	10.00	61	TTGGCTCCCAGCGCCGCAAA// GATCCAGAGCGATTTGACGGCA

**Repeat refers to the repeat unit of the simple sequence repeat, and comp. indicates a compound repeat, consisting of more than one repeat type.*

*** Bin indicates chromosomal location*

3.6 Statistical analysis

Analysis of variance was performed for all measured morphological traits in order to test the significance of variation among inbred lines. The standardized traits mean values (means of each trait was subtracted from the data values and the result divided by the standard deviation) were used to perform principal component and cluster analyses using PAST 2000 software (Ryan *et al.*, 1995). To group the lines based on morphological similarity, cluster analysis was conducted on the Euclidean distance matrix with the unweighted pair group method based on arithmetic averages.

For SSR data analysis, gel photographs were scored manually and repeated twice to limit out errors in scoring. The bands were binary coded by **1** or **O** for their presence or absence in each genotype respectively. Estimates of similarity among all the lines were calculated from the matrices in the form of similarity units and expressed as Euclidean genetic distance (Hintze, 1998).

Cluster analysis was performed to generate a dendrogram using the unweighted pair group method with arithmetic average (UPGMA) as implemented in the popgene version 2.1.

CHAPTER FOUR

4.0 Results

4.1 Morphological traits

Morphological variation was observed among the genotypes studied. Silk varied from deep purple (very strong-score of 5) in EM12-210 (Fig 3 (i)) to deep white (absent-score of 1) in CML206 (Fig.3 (ii)) and purple (strong-score of 4) in CML 312 (Fig.3 (iii)). The analyses of variance reveal highly significant differences ($P<0.001$) among the inbred lines for most of the traits. This suggests that there is a high degree of phenotypic differences among the lines (Appendix 5).

i) CML 206 ii) CML 312 iii) EM12-210

Fig. 3 a) Intensity coloration of the silk (i) **CML206**-Absent (score of 1), (ii) **CML312**-strong (score of 4), (iii) **EM12-210**-Very strong (score of 5)

Key

Very strong (deep purple) - score of 5

Strong (purple) - score of 4

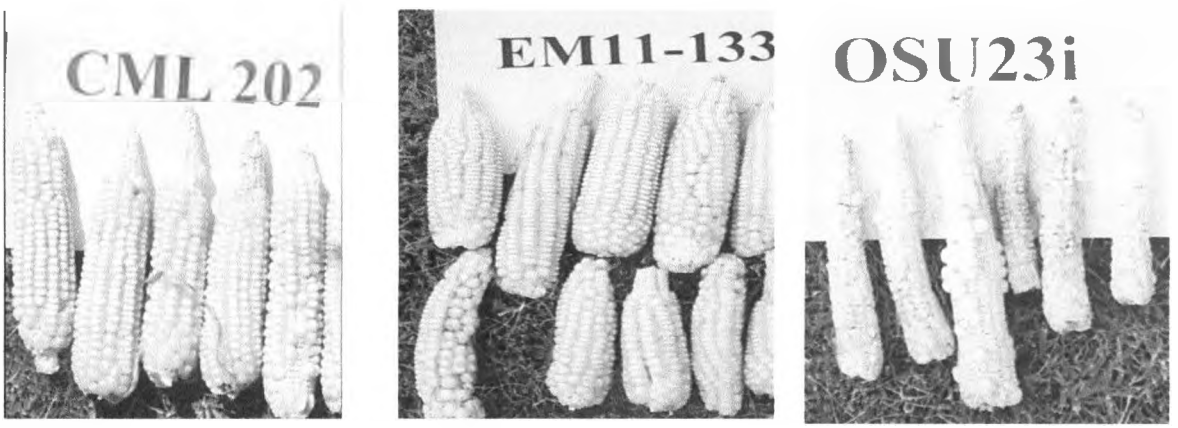
Medium (light purple)-score of 3

Weak (Slight colour)-score of 2

Absent (no colour)-score of 1

i) CML 206 ii) CML 312 iii) EM12-210

Fig 3 b) Anthocyanin coloration of glumes (general) (i) **CML206**-absent (score of 1), (ii) **CML312**-weak (score of 2), (iii) **EM12-210**-strong (score of 4), Angle between main axis and lateral (all medium-45-60%) branches (score of 2), Attitude of lateral branches (all straight) (score of 1) and number of primary lateral branches



i) CML 202 ii) EM11-133 iii) OSU 23i

Fig 3 c) Ear shape, Colour of dorsal side of grain, type of grain, ear aspect and ear diameter (i) **CML202**-Cylindrical (score of 3), white (score of 1), (Semi-dent score of 4), good (score of 4), (ii) **EM11-133**-conical cylindrical (score of 2), yellowish-white, flint (score of 1), good (score of 4), (iii) **OSU23i**-Cylindrical (score of 3), white (score of 1), flint (score of 1), poor (score 1) respectively).

The ten maize lines differed significantly for grain yield (GY), 200-seed Weight (200 SWT), days to tasseling (DTT), days to silking (DTS), plant and ear height (PHT &EHT), ear length (EL), length of peduncle and ear diameter (Appendix 5). The mean yield was 1.784 ton/ha with lower and upper 95% confidence limit of 0.22 and 5.37 respectively (Table 4). EM11-133 had the highest yield of 5.37 ton/ha while CML 312 had the lowest with 0.22 ton/ha.

The weight of 200 seeds (200SWT) ranged from 41.52 grams to 104.67 grams with grand mean of 79.8 grams and none of the ten inbred lines that fall below or above the 95% confidence limit of 0.619 and 2.95 respectively. When the days to anthesis and silk emergence (50%) were considered, CML 444 lied below the lower 95% confidence limit with 83 days for anthesis and 93 days for silking while CML206 was above the upper 95% confidence limit with 160 days for anthesis and 124 days for silking. This signifies good necking between anthesis and silk emergence therefore low anthesis and silking interval (ASI).

The plant and ear height expressed a mean of 136.5 cm and 73.6cm respectively. CML 206 had the lowest plant height of 93 cm while EM12-210 had the highest with 166cm. CML 442 had the lowest ear height of 42cm while EM11-133 had the highest of 105 cm. Em11-133 and EM12-210 lay above the upper 95% confidence limit for both plant and ear height. CML 204 lied below the lower 95% confidence limit with 93cm and 47cm for plant and ear height (Table4).

Table 4 .Summary statistics of the morphological traits measured in ten KARI-Muguga maize inbred lines

Trait	Mean	SD	SEM	Lower 95% conf.limit	Upper 95% conf.li
Anthcyanin colouration of sheath	1.8	1.32	0.42	0.86	2.74
Angle between leaf blade and stem	1.1	0.32	0.10	0.87	1.33
Attitude of leaf blade	2.1	0.57	0.18	1.69	2.5.60
Stem degree of zig-zag	1.5	0.85	0.27	0.89	2.12
Anthocyanin colouration of brace roots	2.6	0.97	0.31	1.91	3.29
Time of anthesis (50%)	103.4	24..25	7.67	86.05	120.75
Anthocyanin colouration of base of glumes	4.8	2.25	0.71	3.19	6.41
Antho colouration of glumes excluding base	2.8	1.03	0.33	2.06	3.54
Anthocyanin colouration of anthers	2.3	0.95	0.30	1.62	2.98
Density of spikelets	5.2	0.63	0.20	4.75	5.65
Angle between main axis and lateral branchesof tassels	1.6	0.67	0.22	1.10	2.10
Attitude of lateral branches of tassels	1.8	0.79	0.25	1.24	2.36
Time of silk emergence (50%)	105.5	15.31	4.84	94.55	116.45
Intensity of anthocyanin colouration of silks	2.9	1.29	0 41	1.98	3.82
Anthocyanin colouration of internodes	2.1	1.10	0.35	1.31	2.89
Leaf width of blade	2	0.67	0.21	1.52	2.48
Length of ear peduncle(cm)	9.57	3.18	1.01	7.30	11.84
Length of husks off tip of ear(cm)	2.6	0.52	0.16	2.23	2.97
Ear length(cm)	13 45	2.63	0.83	11.57	15.33
Ear diameter(cm)	3.60	0.60	0.19	3.17	4.03
Ear shape	2.30	0.68	0.21	1.82	2.78
Number of rows of grain per ear	120	0.94	0.30	11.33	12.67
Type of grain	1.50	0.71	0.22	0.99	2.01
Plant height(cm)	136.50	22.09	6.99	12.70	152.30
Ear height(cm)	73.60	17.48	5.53	61.10	86.11
200SWT	79.80	1.63	0.52	0.62	2.95
YIELD(tonha^{-1})	1.78	1.63	0.52	0.22	5.37

Key: SD- Standard deviation, SEM.-standard error of the mean

4.2 Morphological variability

The eigenvalues and eigenvectors of correlation (normalized by variance-covariance) matrix were calculated. Correlation matrix was used rather than variance-covariance matrix since the variables were measured in different units. This implies normalizing all variables using division by their standard deviations.

The first five principal components (PCs), which had eigenvalues higher than 1.5 explained a total cumulative percentage of 85.62% of the phenotypic variation (Table 5). In the first PC, which explained 47.09% of the total variation, the most important traits were: plant and ear height, days to silking and tasseling and 200 SWT. In the second PC, which explained 12.63% of the total variation, predominant traits were; days to silking and tasseling. The third PC explained 11.54% of the total variation, the most important traits were; plant and ear height and anthocyanin coloration of the brace roots. The fourth PC explained a total of 8.48% of the total variation; the predominant traits were anthocyanin coloration of the leaf sheath, attitude of the leaf blade, anthocyanin coloration of the brace roots, days to silking, number of rows per ear and plant height. The firth PC explained a total of 5.80% of variation with days to silking, length of husks off tip of ear and ear length been the main contributors.

Table 5.Eigenvectors, eigenvalues and cumulative percentage of variation explained by the first five principle components (PC) after assessing morphological traits in ten maize inbred lines.

Principle components	PC1	PC2	PC3	PC4	PC5
Anthcyanin colouration of sheath	-0.4549	-0.0006	0.17951	0.4591	-0.0165
Angle between leaf blade and stem	-0.4657	0.03298	0.03103	0.17172	0.08459
Attitude of leaf blade	-0.4569	0.01484	-0.0556	0.2310*	-0.167
Stem degree of zig-zag	-0.4518	0.02780	0.01714	0.2037*	-0.4173
Anthocyanin colouration of brace roots	-0.4467	0.07816	0.04801	0.2479*	-0.4372
Time of anthesis (50%)	2.263*	3.7457*	0.4296*	-1.0089	-2.5849
Anthocyanin colouration of base of glumes	-0.3830	-0.1584	-0.1105	-0.0824	-0.1078
Antho colouration of glumes excluding base	-0.4385	-0.0185	0.01825	0.1089	0.18133
Anthocyanin colouration of anthers	-0.4520	-0.0059	0.02367	0.2127	-0.0871
Density of spikelets	-0.3786	0.00387	0.01495	0.2156	-0.1016
Angle between main axis and lateral branchesof tassels	-0.4539	-0.0123	-0.0540	0.0524	-0.4627
Attitude of lateral branches of tassels	-0.4437	0.01292	-0.0285	0.1455	-0.3135
Time of silk emergence (50%)	2.3277*	1.9833*	-0.3734	0.8511*	3.789*
Intensity of anthocyanin colouration of silks	-0.4336	-0.0341	0.08618	0.1594	0.19756
Anthocyanin colouration of internodes	-0.4466	-0.0356	0.1542	0.3005	-0.0836
Leaf width of blade	-0.4606	0.01051	0.06043	0.0587	0.20142
Length of ear peduncle(cm)	-0.2606	-0.1588	-0.0136	-0.1726	0.5334*
Length of husks off tip of ear(cm)	-0.4492	0.02142	0.03058	0.4172	0.3258*
Ear length(cm)	-0.1523	-0.1801	-0.6109	-0.2351	-1.0176
Ear diameter(cm)	-0.4309	-0.0023	0.32937	-0.0308	-0.3787
Ear shape	-0.4386	0.08673	0.00607	0.1039	-0.0843
Number of rows of grain per ear	-0.2226	0.19844	-0.1714	0.6081*	-1.1023
Kernels per row	-0.3575	0.13556	0.39203	-0.9274	1.947
Type of grain	-0.4546	0.03995	0.05008	0.0696	-0.2026
Plant height(cm)	3.187*	-2.5125	1.5079*	2.8738*	-0.8472
Ear height(cm)	1.3715*	-1.7996	2.3486*	-3.9996	0.3069*
200SWT	1.6616*	-1.5235	-4.5932	-1.2999	-0.3216
YIELD(ton/ha)	-0.4630	-0.0207	0.13775	-0.2563	0.69554
Eigenvalue	14.5975	3.9160	3.5772	2.6277	1.7942
Individual percentage	47.09	12.632	11.539	8.477	5.798
Acculated variation %	47.09	59.721	71.361	79.837	85.624

*Traits that are corresponding to underlined numbers are the most significant traits that contribute much of the variation in each PC

A dendrogram generated from the standardized morphological data (Figure 4) revealed three clusters. The first cluster contained five inbred lines all from CIMMYT (CML395, CML442, CML206, CML312 and CML204). All the lines had days to silking

above 105 days and an average yield of 0.702 $Tonha^{-1}$. CML 395, CML 442 and CML 312 were sub-clustered together. The three inbred lines had their days of anthesis ranging from 100 days to 110 days and that of silking ranging from 104 days to 115 days. The second sub-cluster consisted of CML206 and CML 204, which had 160 days and 102 days to anthesis, 124 days and 107 days to silking respectively (Appendix 5).

The second cluster contained four inbred lines, two from CIMMYT (CML202 and CML444) and two from KARI (EM11-133 and EM12-210) with an average plant height of 153cm. EM11-133 had the highest yield of 5.37 Ton/ha with medium anthocyanin coloration. EM12-210 had an average yield of 1.9 Ton/ha and highest scores for anthocyanin coloration (strong-4 and very strong-5). The third cluster contained one inbred line whose origin is Ohio State University (OSU23i) with a plant and ear height of 130 cm and 67 cm, respectively. It is the only line whose days to silking and tasseling were below 80 days (Appendix 5).

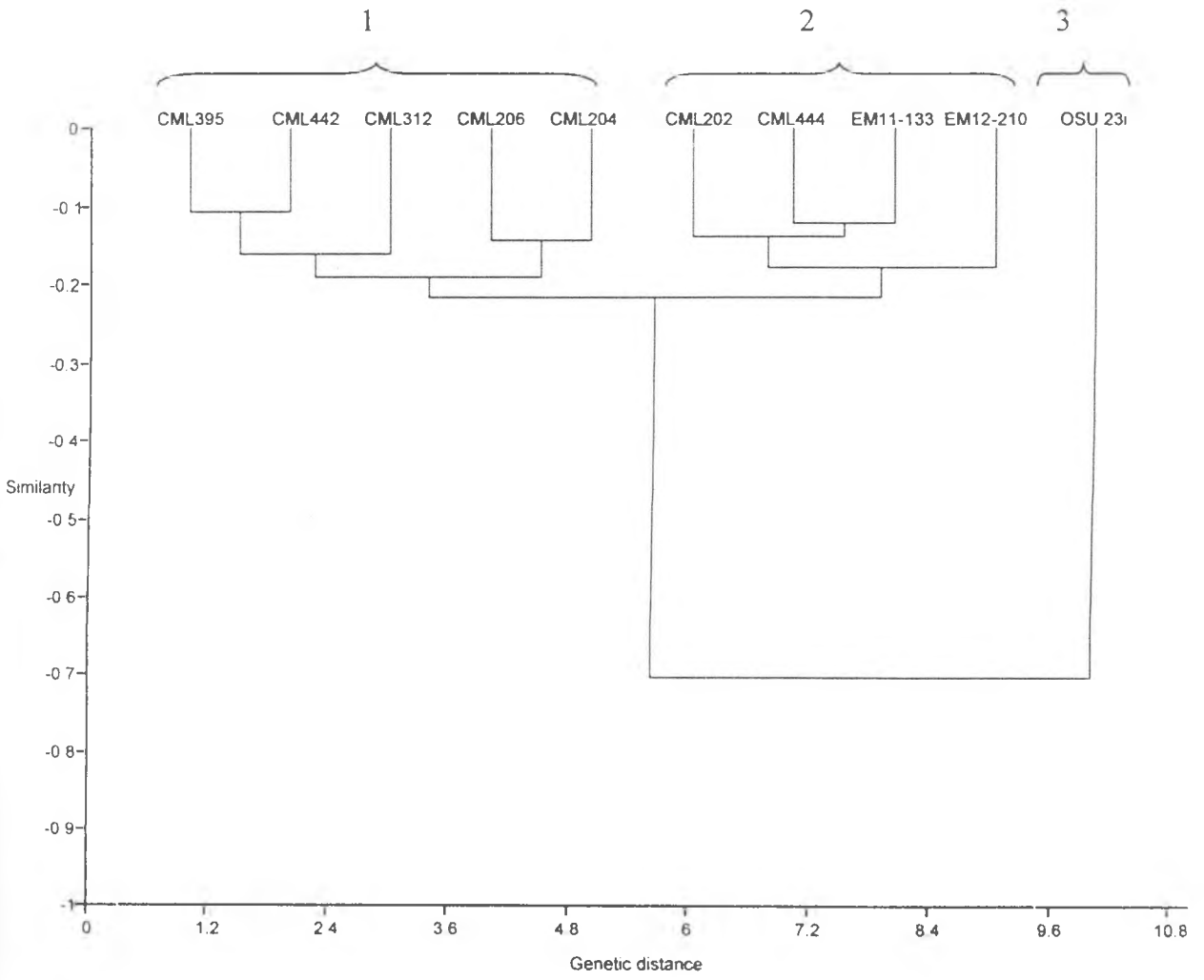


Fig: 4 Dendrogram of the ten maize inbred lines derived by UPGMA from the similarity matrix of the morphological data.

4.3 DNA analysis

Genomic DNA was extracted from randomly selected individual plants each representing a variety of the inbred lines is shown in Fig 5. Since there was no shearing genomic DNA was free from the RNA, was stable and equally of good quality.

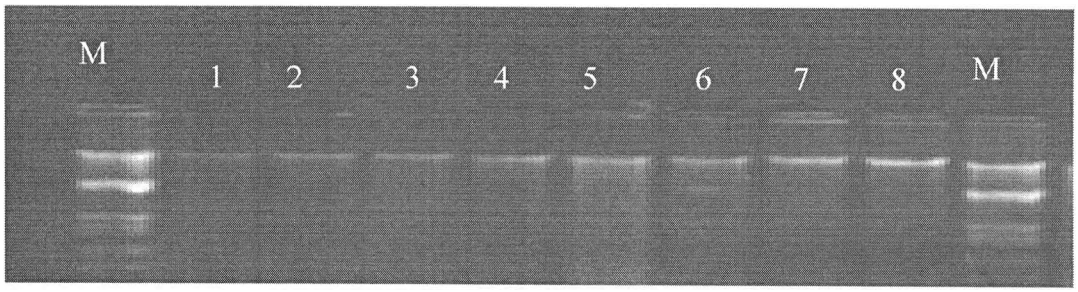


Fig.5 Genomic DNA resolved with 0.8% (w/v) agarose and ran at 100V for 50 minutes (1μl of each sample loaded)

Lane M-500ng concentration marker, **1**-EM11-133, **2**-EM12-210, **3**-OSU23i, **4**-CML395, **5**-CML202, **6**-CMl442, **7**-CML44, **8**-CML206 and **9**-CML312

4.3.1 DNA quantification using spectrophotometer

The concentration of the genomic DNA extracted from the ten inbred lines ranged from 17-159 µg/ml (Appendix 3).

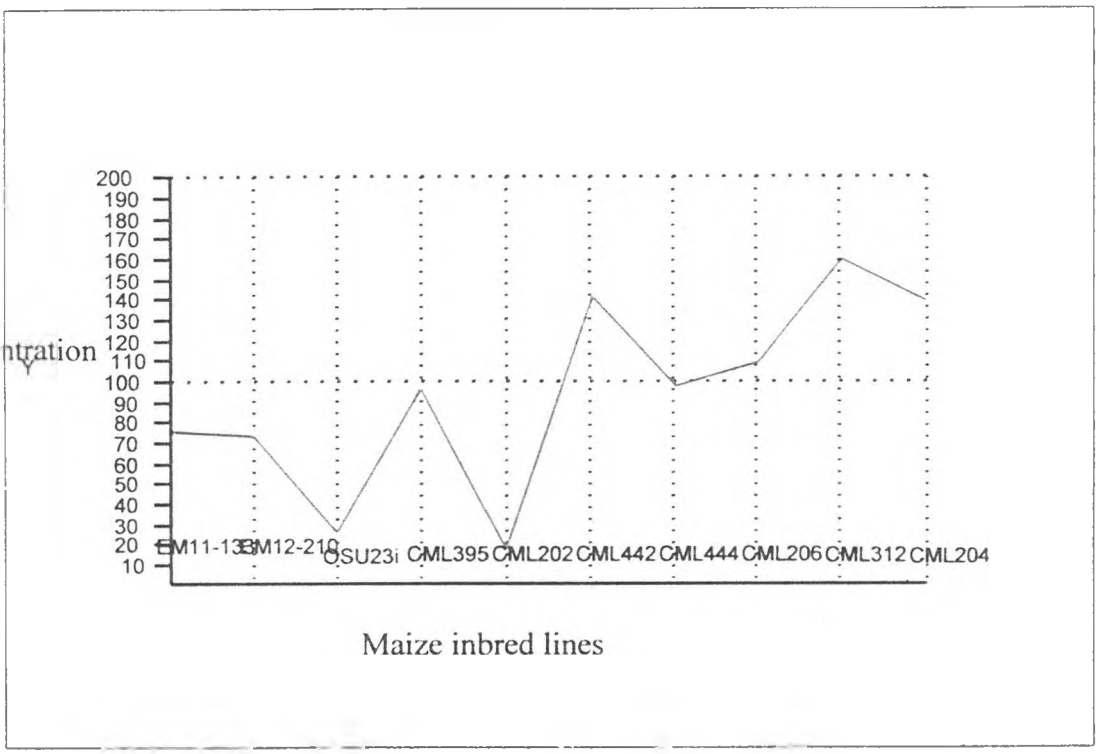


Fig. 6: The distribution of DNA concentration of samples from the ten inbred lines

4.3.2 DNA purification and purity checks

The quality of genomic DNA extracted was checked for contaminants using spectrophotometer readings at wavelengths of 260nm and 280nm. The ratio of the optical densities (OD) ranged from 1.683 to 1.938 (Appendix 3). This was indicative of relatively pure DNA, not contaminated with proteins, RNA, and carryover chemicals from extraction buffers and reagents.

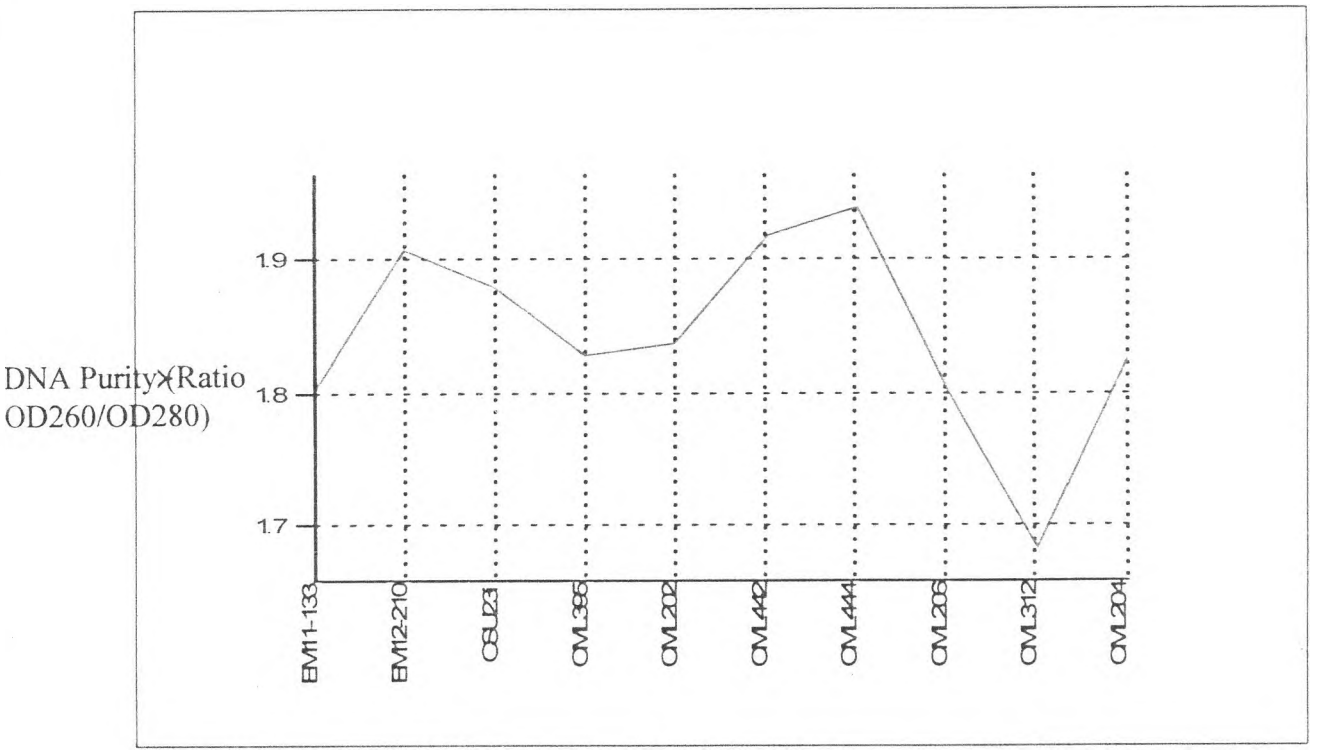


Fig. 7: Variation in the DNA Purity.

Nine samples had an OD ratio of between 1.8 and 2.0, which is the standard required DNA purity. Genomic DNA from CML 312 had an OD of 1.68 which meant that it had contamination with protein. The DNA was re-precipitated with ice-cold alcohol for purification.

4.4 SSR analysis

SSR bands were scored manually for each individual maize line from the gel photograph. The bands were recorded as discrete characters, presence '1' or absence '0' which was repeated twice to check the reproducibility of the bands and minimize the scoring error (Table 6). A total of 28 bands were amplified in the ten maize lines using the 14 SSR primers. None of the SSR loci revealed two alleles (two bands) in single inbred line. The size of SSR fragments ranged from 100 to 300 bp. The representatives of microsatellite bands resolved on a 2.0% agarose (w/v) gel are shown in Fig.8 A-D.

Table 6. Distribution of bands in the ten inbred maize lines using 14 SSR primers

Markers	Em11-133	EM12-210	OSU 23i	CML395	CML202	CML442	CML 444	CML 206	CML 312	CML 204
umc1122	1,1	0,0	1,0	1,1	1,1	0,0	1,1	0,0	0,0	0,0
phi339017	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	0,0
phi101049	1,1	0,0	0,0	1,1	1,1	1,1	1,1	0,0	1,1	0,0
phi127	0,0	0,0	1,1	1,1	1,1	1,1	1,1	0,0	1,1	1,1
phi053	1,1	1,1	1,1	0,0	0,0	0,0	0,,0	1,1	0,0	0,0
phi029	1,1	0,0	0,0	1,1	1,1	1,1	1,1	1,1	1,1	0,0
phi331888	1,1	0,0	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1
phi423796	1,1	1,1	0,0	1,1	0,0	1,1	0,0	1,1	0,0	1,1
phi328175	1,1	1,1	1,1	0,0	1,1	1,1	1,1	1,1	1,1	1,1
phi034	1,1	0,0	1,1	1,1	0,0	1,1	1,1	1,1	0,0	0,0
phi100175	1,1	0,0	0,0	1,1	1,1	1,1	1,1	0,0	1,1	0,0
phi233376	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1	1,1
phi059	1,1	0,0	1,1	1,1	0,0	0,0	0,0	0,0	0,0	0,0
phi041	1,1	0,0	1,1	1,1	1,1	0,0	0,0	0,0	0,0	0,0

Key: 1- presence of band; 0- absence of band

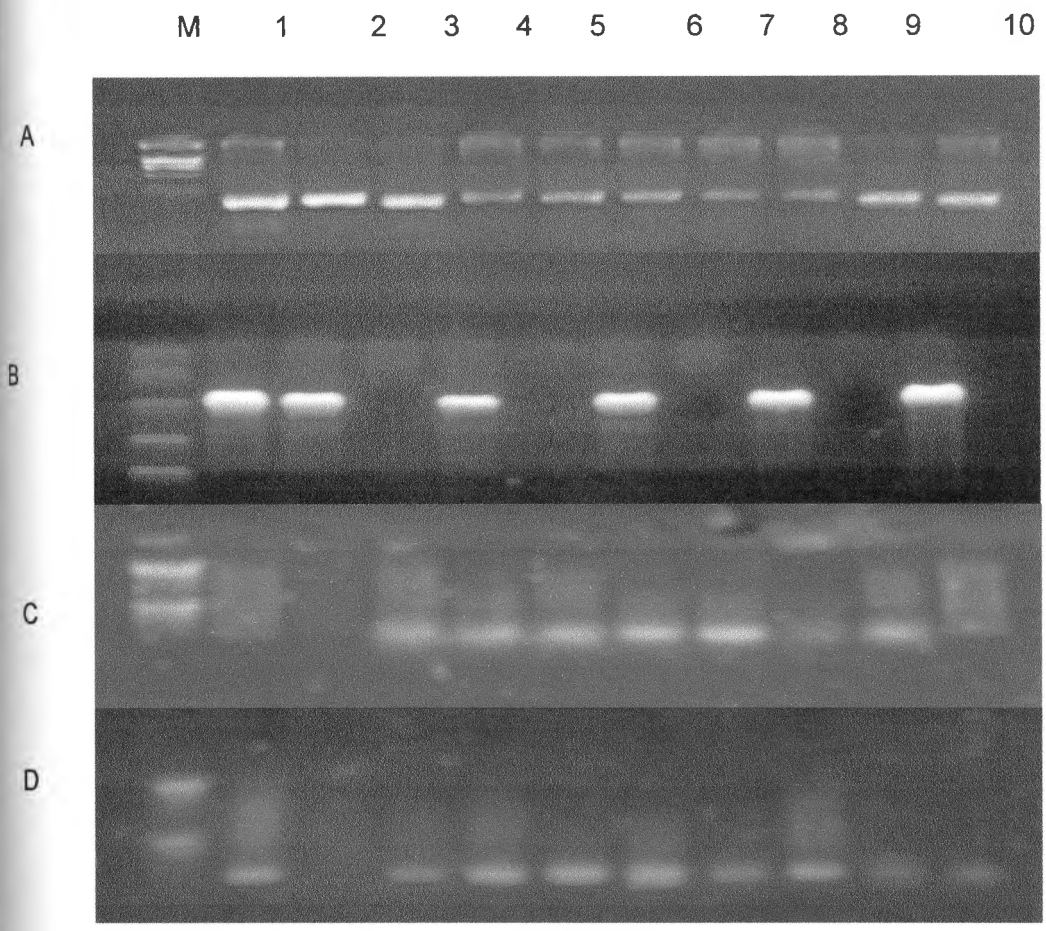


Fig. 8 Amplification of genomic DNA from ten mid altitudes –adapted maize inbred lines using SSR primers (A) primer set phi 233376 (B) phi 423796, (C) phi 127 and (D) phi 331888. Lane M-100bp ladder, 1-EM11, 2-EM12, 3-OSU23i, 4-CML395, 5-CML202, 6-CML442, 7-CML444, 8-CML206, 9-CML312 and 10-CML204.

4.5 Genetic distance

Unweighted pair group method (UPGM) was used to produce the dendrogram on the basis of 14 SSR primers and the ten lines to study the overall genetic distance between the lines. The Dice's genetic identity (Nei and Li, 1997) was also calculated to correlate the genetic distance (GD) among the inbred lines (Table 7). The GD ranged from 0.154 to 1.54. The most diverse lines were EM12-210 and CML 395, EM11-133

and CML 204 and CML202 and CML 204, all with a GD of 1.54. EM11-133 and CML 395. CML 444 and CML442 and CML 206 and CML 312 with closely related with a GD of 0.154 (Table 7).

The resulting dendrogram indicated that most of ten lines could be distinguished and clustered into three groups (Fig. 9).

Cluster 1 consisted of three CIMMYT lines (CML206, CML312 and CML204), which had no amplification with umc 1122. CML206 and CML312 were sub-clustered together with a GD of 0.3365 compared to that of CML204 of 0.5714. The two inbred lines (CML206 and CML 312) had same amplification with phi339017, phi029 and phi331888 with a total number of bands. They also had similar morphological traits such as anthocyanin coloration of sheath, attitude of thee leaf blade, anthocyanin coloration of the brace roots, attitude of the lateral branches of the tassel and the length of ear peduncle.

In cluster II, grouped consisted of CML 395, CML 202, CML 442 and CML 444) with two sub-divisions, which could have been contributed by phi041. Their genomic DNA was amplified with phi101049 and phi100175 but with no amplification with phi053. They also had similar traits such as angle between leaf blades with a score of 1, stem, degree of zigzag for the stem and an average of 12 rows of grain pre ear. CML395 and CML 202 were sub-clustered together, with amplification with phi041. They had also close similar morphological traits such as days to silking, 115 and 116 days respectively, tasseling, length of ear peduncle, ear length, and ear diameter, shape of the ear plant and ear height and recording a GD of 0.337. CML444 and CML442 were sub-clustered together with no amplification with phi041.They also had morphological similar traits

such as coloration of the tassel glumes (excluding the base), coloration of the anthers, and attitude of the lateral branches of the tassels, length of husks off tip of ear and ear shape and recording a GD of 0.154.

Cluster III contained two KARI lines (EM11-133 and EM12-210) and OSU line. The two KARI lines were sub-grouped together suggesting a common ancestral that is Embu composite. EM11-133 was the only inbred line that amplified with all the fourteen SSR primers. The three inbred lines amplified with phi053 and phi328175. They also showed similar morphological traits such as density of spikelets, attitude of lateral branches of the tassel and the length of ear peduncle. The three inbred lines differed significantly on the anthocyanin coloration of sheath, brace roots tassel, anthers and internodes which was either strong with a score of 4 or very strong with a score of 5 for EM12-210. EM11-133 had the highest yield of 5.37 Ton/ha and ear height of 105cm.

Table 7: Genetic Identity (ID) (above diagonal) and Genetic distance (GD) (below diagonal) of the ten maize inbred lines generated from the fourteen SSR markers as per Nei (1972) Am. Nat. 106:283-292)]

Pop ID	EM11-133	EM12-210	OSU 23i	CML 395	CML 202	CML 442	CML 444	CML 206	CML 312	CML 204
EM11-133	****	0.357	0.785	0.857	0.714	0.642	0.785	0.642	0.642	0.214
EM12-210	1.02*	****	0.571	0.214	0.5	0.428	0.428	0.714	0.428	0.714
OSU 23i	0.241	0.559	****	0.642	0.5	0.428	0.571	0.714	0.428	0.428
CML 395	0.154**	1.54*	0.441	****	0.714	0.642	0.785	0.5	0.642	0.214
CML 202	0.336	0.693	0.693	0.336	****	0.785	0.928	0.642	0.785	0.5
CML 442	0.441	0.847	0.847	0.441	0.241	****	0.857	0.714	1	0.571
CML 444	0.241	0.847	0.559	0.241	0.074	0.154**	****	0.714	0.857	0.428
CML 206	0.441	0.336	0.336	0.693	0.441	0.336	0.336	****	0.714	0.571
CML 312	0.441	0.847	0.847	0.441	0.241	0.273	0.154**	0.336	****	0.571
CML 204	1.54*	0.336	0.847	1.54*	0.693	0.559	0.847	0.559	0.559	****

*The genetic distance indicates the lines, which are diverse from each other

** The genetic distance indicates the lines, which are closely related

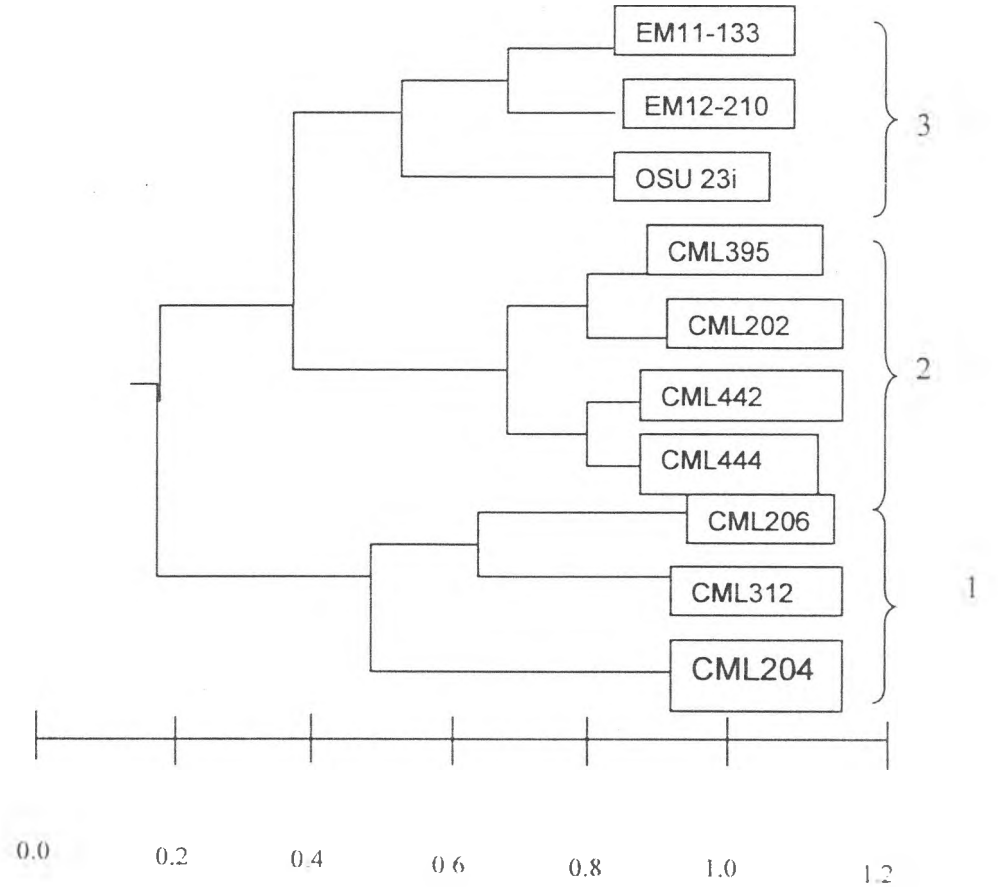


Fig. 9 Dendrogram generated for the ten maize inbred lines using fourteen SSR markers based on Nei's (1972) Genetic distance: Method = UPGMA

CHAPTER FIVE

5.0 Discussion

All the fourteen SSR primers used revealed one band during amplification, which may have resulted from the co-dominant nature of the SSR primers unlike the more than two bands that were reported by Bantte and Prasanna 2003. This indicated that the inbred lines may be homozygous, had no mutation at specific SSR loci, or amplification of similar sequences in different genomic regions due to duplication. This could also indicate that the lines are at advanced generation of inbreeding. Minimum genetic distance revealed a (<0.154) confirming the power of SSR markers to distinguish between closely related inbred lines (Smith *et al*., 1997).

The dendrogram constructed using the UPGMA clustering algorithm grouped the inbred lines into three clusters, which may be generalized into two main clusters in both morphological traits and SSR markers analysis. When both morphological traits and SSR analysis are compared, they revealed evidence of associations related to their origin and pedigree records as seen with EM11-133 and EM12-210, CML204 and CML206 (Fig.4 and 9). This is in agreement with earlier investigators (Smith *et al*., 1997; Senior *et al*., 1998 and Reif *et al*., 2003), who demonstrated the correspondence of SSR marker distance with pedigree information in maize. This is true for the case of CML442 and CML444, which also had the lowest GD of 0.154. Alternatively, grouping of the inbred lines could have been based on the adaptation regimes thus few clusters as seen with the CML lines, which are adapted to the mid-altitude regions.

Despite this, the mixed group of EM11-133, EM12-210 and OSU23i shows some tendency of fluctuation from the available pedigree or contamination during hand pollination. This could be true since the ten inbred lines under investigation have been advanced and maintained at the same breeding center (KARI-Muguga).

In addition, factors such as number of SSR loci and repeat types and, the methodologies employed for detection of polymorphic marker have been reported influence allelic differences. In this work, the average number of alleles (2.0) and the number of SSR loci (14) used to screen the ten inbred lines were considerably lower than those reported previously in maize. Warburton *et al.*, (2002) with 85 SSR loci found an average of 4.9 alleles per locus and Vaz Patto *et al*., 2004 obtained an average of 5.3 alleles per locus using 80 SSR loci. These investigators also used the metaphor gel to screen the microsatellites loci in maize.

In this work, agarose gel electrophoresis was used for the screening of the SSRs. Compared to polyacrylamide gel or automated analysis; this is the most appropriate technology for routine is a less costly and more widely available gel system. However, it is possible that an automated detection system would have been be able to resolve allelic variation better than gel electrophoresis analysis, and consequently, the number of alleles obtained would even be higher than that reported in this study. This may be particularly important where >20 SSR loci containing dinucleotide repeats have been applied and whose amplification products are in 130 to 200 base pair range, because PCR products differing by two base pairs cannot be resolved with agarose gel (Senior *et al.*,1998; Sibov *et al.*,2003).

All the CMLs clustered together that is cluster 1 and 2 in both morphological and molecular clustering. This indicates that the CMLs are closely related or have the same parental source with GD ranging from 0.154 to 1.54. This could be the reason why CML206, CML312 and CML204 were clustered together in both morphological and molecular analysis. CML442 and CML444 could have been extracted from closely related populations or populations of same parental source and selected at different levels during the selection process. This is also revealed by EM11-133 and EM12-210, which share same parental source as well as developed from the same breeding center (KARI Embu).

The UPGMA dendrogram for morphological and SSRs (Figs 4 and 9) analysis also clustered CML202 and CML 444 together. This indicates the close similarities and common parental sources and only differs in height, yield and resistance to MSV, where CML202 is tolerant to MSV with a score of 1.5, plant and ear height of 140 cm and 80 cm, respectively and a yield of 2.5 Ton/ha . CML444 is susceptible to MSV with a score of 2.5, plant and ear height of 144 cm and 81 cm, respectively and yield of 2.0 Ton/ha (CIMMYT 2004). CML 202 was the first evaluated and released as tolerant to MSV. Further development and evaluation of CML202 could have resulted to CML 444 which is of drought tolerance and low nitrogen efficiency use.

EM11-133 and EM12-210 were also clustered together in the two dendrograms suggesting the close similarities between them. The two inbred lines originated from Embu composite in 1964. The two lines are known to be of good combining ability for yield but highly susceptible to major disease constraints such MSV, rust, blight and GLS and only differ in anthocyanin coloration of the leaf sheath, brace roots, glumes

excluding the base, anthers, internodes and stem degree of zigzag which is either absent or weak for EM11 133 but strong for EM12-210. This is a good demonstration of agreement between the SSR markers with pedigree information in maize.

CML206 and CML204 were also clustered together in the two UPGMA dendrograms (Fig.4 and 9). The two lines have close morphological characteristics such as ear height, ear length and anthocyanin coloration of the anthers. The two lines also belong to the same series; that is they were developed and confirmed as inbred lines at the same time. This indicates that the lines were extracted from the same population or related population as with the case of CML lines.

OSU23i was clustered together with EM11-133 and EM12-210 in molecular analysis (Fig 9) unlike in morphological analysis (Fig 4). This suggests that the line could be of the same genotype as the EMs and differ from them from its medium anthocyanin coloration and high resistance to MSV or contaminated during pollination and only differ in yield, plant and ear height and disease resistance. OSU23i is of high resistance to MSV with a score of >2.0.

Morphological traits are relatively less reliable for precise discrimination of closely related lines and analysis of their genetic relationship as revealed in figure 4 where OSU 23i clustered alone. This could be due the incomplete pedigree records sometimes encountered in the breeding programs, contamination during hand pollination followed by bulking of the individual line or advancement of the line. This may result to segregating lines leading to unstable genotype. This is commonly evidenced when comparing molecular results with classification based on pedigree information as revealed in this study.

Despite this limitation, morphological traits are useful for preliminary evaluation because it is fast, simple, and cheap and can be used as a general approach for assessing genetic diversity among morphologically distinguishable lines such as the ten inbred lines studied.

5.1 Conclusion

This study indicated that the fourteen SSR markers relatively separated the ten inbred lines into different clusters, which generally agreed with the source of origin and time of development e.g. CML206 and CML204 were also clustered together in morphological and molecular analysis..

Despite the high morphological diversity expressed by the ten maize lines, their genetic similarities and differences can be resolved with the combination of both techniques.

Since the KARI-Muguga inbred lines are maintained through hand pollination, this could be the source of contamination especially between EM11-133, EM12-210 and OSU 23i.

Genetic analysis is an alternative tool for resolving genetic diversity of maize lines that are morphologically diverse.

5.2 Recommendations

1. There is need to screen the ten inbred lines with more SSR markers to reveal the genetic differences of the clusters such as CML 202 and CML 395, EM11-133, EM12-210 and OSU 23i whose grouping contradicts lines of origin.
2. A better method for line management and maintenance should be adapted to avoid line contamination such as ear to row.

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7.0 APPENDICES

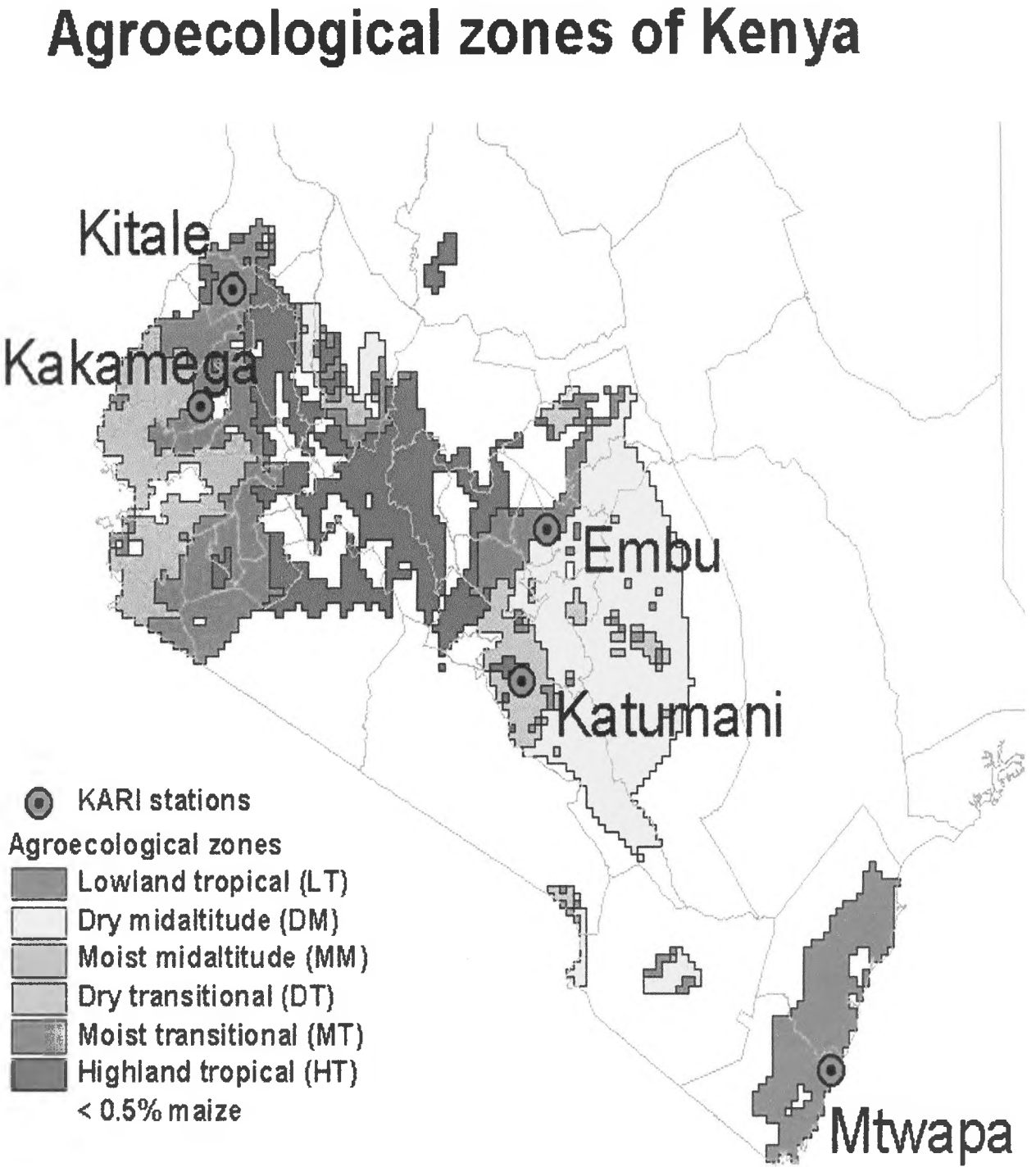
Appendix 1

The major maize varieties grown in Kenya

MAIZE VARIETIES AT A GLANCE			
VARIETY	AVERAGE YIELD/HA	ALTITUDE RANGE (M)	DAYS TOMATURITY
H6213	128	1700-2100	5-7 Months
H6212	128	1700-2100	5-7 Months
H6210	123	1700-2100	5-7 Months
H9401	118	1700-2100	5-7 Months
H629	118	1700-2400	5-7 Months
H628	113	1500-2100	5-7 Months
H627	108	1500-2100	5-7 Months
H626	109	1500-2100	5-7 Months
H625	98	1500-2100	5-7 Months
H614	94	1500-2100	5-6 Months
Pwani Hybrid 1	40	1-1500	3-4 Months
Katumani Composite B	33	1000-1900	2-3 Months
DLC1	27	1000-1900	2-3 Months

Source: Kenya Seed Company

Appendix 2 Agro-climatic zones map of Kenya



Source: www.kari.org

Appendix 3 Quantification of DNA for PCR analysis

Sample	Concentration (*ng/ml*)	Ratio(260/280)
EM11-133	75	1.801
EM12-210	73	1.907
OSU23i	26	1.879
CML395	96	1.828
CML202	17	1.836
CML442	141	1.918
CML444	97	1.938
CML206	109	1.801
CML312	159	1.683
CML204	139	1.823

Appendix 4: Stock solutions

a) CTAB extraction buffer1

Stock solution	Final concentration	200ml
dH_2O		130ml
1M Tris-PH 7.5	100mM	20ml
5M NaCL	700ml	28ml
0.5 M EDTA-PH 8.0	50mM	20ml
CTAB[2] 1%		2g
14M BME[3]		2ml

1 Freshly made, warm buffer to 65oc was prepared before adding the CTAB and BME.

2 CTAB= Mixed alkyltrimethyl-ammonium bromide

3 BME (B-mercaptoethanol) added just prior to use, under a fume hood

b) WASH 1 76%EtOH, 0.2M NaOAC

Stock	200ml
Absolute EtOH	76ml
2.5M NaOAC	8ml
dH_2O	32ml

c) WASH 2 76% EtOH, 10mM NH_4OAC

Stock	200ml
Absolute EtOH	152ml
1M NH_4OAC	2ml
dH_2O	46ml

d) CHLOROFORM: OCTANOL: 24:1

Stock	200ml
Chloroform	192ml
Octanol	8ml

Appendix 5: Morphological Data for the ten maize inbred lines

Agronomic traits	Inbred line and the agronomic trait score									
	EM11-133	EM12-210	OSU23i	CML 395	CML 202	CML 442	CML 444	CML 206	CML 312	CML 204
Anthcyanin coloration of sheath	1	5	2	1	1	1	2	1	1	3
Angle between blade and stem	1	1	2	1	1	1	1	1	1	1
Attitude of blade	2	2	3	2	2	3	2	2	2	1
Degree of zig-zag	1	3	3	1	1	1	1	1	1	2
Anthocyanin colouration of brace roots	1	3	3	2	2	3	2	4	4	2
Time of anthesis (50%)	107	88	68	110	114	100	83	160	102	102
Anthocyanin colouration of base of glumes	5	5	7	4	7	5	6	1	7	1
Antho colouration of glumes excluding base	3	4	3	3	4	3	3	1	3	1
Anthocyanin colouration of anthers	2	3	3	3	2	2	2	1	4	1
Density of spikelets	5	5	5	5	5	5	7	5	5	5
Angle between main axis and lateral branches	2	1	3	1	1	2	1	1	2	2
Attitude of lateral branches	2	2	2	1	1	3	3	1	1	2
Time of silk emergence (50%)	114	97	71	115	116	104	93	124	114	107
Intensity of anthocyanin colouration of silks	2	5	3	3	3	3	2	1	5	2
Anthocyanin colouration of internodes	3	4	3	1	1	1	2	1	2	3
Width of blade	3	2	2	2	2	2	3	2	1	1
Length of peduncle	10	9	8	11	10.3	7.7	18	7	7	8
Length of husks off tip of ear	2	3	2	2	3	3	3	2	3	3
Length	16	12	16	14.8	13	17	16	9.5	11	10
Diameter	3.3	4.1	4	3.1	3.2	0	4.6	4.3	3.5	3
Shape	2	1	2	3	3	2	2	3	2	3
Number of rows of grain	10	12	12	12	12	12	14	12	12	12
Kernels per row	11	4	3	4	14	3	8	7	1	5
Type of grain	1	1	2	1	2	1	3	2	1	1
Plant height	158	166	130	130	133	114	156	93	150	135
Ear height	105	88	70	67	70	42	82	47	75	55
200SWT	88	71	84.4	105	79.6	95	88	41.5	84	62
YIELD	5.4	1.9	1.07	0.76	2.15	0.7	3.8	0.75	0.2	1.1