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Increasing line combining ability and gray leaf spot resistance in maize by integrating conventional with DNA marker technology

by

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CO-PROMOTOR: Dr. N.G. Lyimo

DECLARATION

I the undersigned hereby declare that this thesis submitted herewith for the degree Doctor of Philosophy to the University of Pretoria, contains my own independent work as hitherto not been submitted for any degree at any other university or faculty.

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Date

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INCREASING LINE COMBINING ABILITY AND GRAY LEAF SPOT RESISTANCE IN MAIZE BY COMBINING CONVENTIONAL BREEDING WITH DNA MARKER TECHNOLOGY

BY

BARNABAS ANTHONY KIULA

SUPERVISOR: Prof. A-M Oberholster
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ABSTRACT

Maize is the staple food for the majority of Tanzanians. However, maize production in the Southern highlands of Tanzania (SHT) is highly reduced by gray leaf spot disease (GLS) caused by the fungus *Cercospora zea maydis*. GLS reduces grain yield, kernel and silage quality. The most common GLS control methods in Tanzania include amongst others; fungicides, crop rotation, field sanitation, host resistance. These methods except host resistance are, however, either expensive or less effective or unsafe to the environment. Furthermore, conventional breeding strategies are not very effective for traits, which are lowly inherited such as GLS resistance. Lastly, to date there are few GLS resistant commercial hybrids in SHT. Thus, this study aimed to produce more commercial GLS resistant hybrids, increase farmers' hybrid choices of growing genetically different GLS insensitive hybrids, which will also provide a constant supply of GLS resistant maize cultivars in case of GLS resistance breakdown due to new GLS pathotypes. This research combined conventional breeding with molecular technologies to increase the efficacy of selecting GLS resistant hybrids and assist breeders in

predicting best inbred combinations for commercial hybrid production. Studies conducted to meet the main aims were on; the prediction of best line combiners and heterosis in Tanzanian maize breeding lines through the use of amplified fragment length polymorphism, (AFLP), an association of AFLPs and the performance of phenotypic traits in maize, evaluation of maize hybrids for gray leaf spot resistance in multienvironments and finally a preliminary study on gray leaf spot PCR-based marker development with the long term objective of implementing cleaved amplified polymorphic markers (CAPS) in a marker assisted selection (MAS) strategy in the SHT maize breeding programme.

Results from the study revealed that pairwise GD (genetic distance) of the lines varied from a GD of 0.13 to 0.5. High coancentry coefficients were exhibited by these lines. Joint data analyses showed that there were tighter associations between line GD and F_1 traits or MPH in the intergroup than in the intragroup crosses. Combined analyses revealed that hybrids 48, 90 and 45 recorded higher stable yields and consistently low GLS scores in multienvironments. Fifteen CAPS marker bands were identified that are putatively linked to the GLS resistant genes.

In summary, it was noted that strong selection during inbreeding programs should be avoided as it reduces germplasm variability. Local landraces/varieties can be improved by introgressing desirable genes into them. AFLP marker system could be effectively used for inbred genetic diversity studies in Tanzania. Intergroup crosses with high GD-MPH should be the main target for commercial hybrid production but field testing of

them is inevitable to confirm their yielding potentials. Intergroups and intragroup crosses with low GD-MPH should be discarded to avoid field costs. Better F_1 hybrid performance predictions can be achieved by integrating inbred GD and F_1 phenotypic data. Hybrids with low GLS/high GLS resistance could be used to produce other breeding populations. Hybrids 45, 48 and 90 can be commercially preleased. Lastly a study to characterize the GLS fungus in the SHT is imperative since information on virulence of isolates is needed for long term breeding strategies against the fungus. Finally, the SHT maize germplasm has potential GLS resistant inbred lines which could be used in the deployment of genes to susceptible lines and in the development of commercial GLS resistant hybrids/open pollinated varieties/doubled haploid hybrids.

Keywords: AFLP, dendrogram, GLS, GD, genetic diversity, germplasm.

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecule variance
APS	Ammonium persulphate
ASAP	Allele specific associated primers
CAPS	Cleaved amplified polymorphic markers
CIMMYT	Centro international de Mejoramiento de Mazy Trigo
CTAB	Cetyltrimethyl ammonium bromide
DAPS	Days after planting to silking
EDTA	Ethylene diamine tetra acetate
G x E	Genotype x environment
GCA	General combining ability
GAS	Gene assisted selection
GD	Genetic distance
GLS	Gray leaf spot disease
LD	Linkage disequilibrium
LE	Linkage equilibrium
MABC	Marker assisted backcrossing
MAS	Marker assisted selection
MI	Marker index
PCR	Polymerase chain reaction
PIC	Polymorphism information content
QTL	Quantitative trait loci
RAPD	Random Amplified polymorphic DNA
RCBD	Randomized complete block design
RFLP	Restriction fragment length polymorphism
SCA	Specific combining ability
SCAR	Sequence characterized amplified region
SHT	Southern highlands of Tanzania
SSR	Simple sequence repeats
STS	Sequence tagged sites
UMC	a molecular marker which is linked to GLS resistant genes in maize

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DEDICATION

I hereby dedicate my thesis to my LORD JESUS CHRIST, the SAVIOUR of the whole human race and THE POWER OF OUR RESSURECTION. In him we live, we walk and without him nothing can be done. So it is only God and God alone who makes all things possible including the accomplishment of this thesis.

CHAPTER 1

GENERAL INTRODUCTION

Maize (*zea mays* L.) is a staple food for millions of people (dietary energy consumption i.e. kcal/person/day) in several African countries, Asia and South America (FAO; 2004). While in the USA and Europe animal feeding is by far the largest user of maize (USDA, 1986). Maize is among the most important coarse grain consumed by both human beings and livestock. In 1996, the world's average maize yield was 4.1 MT/ha, while in Africa in the same year; the average production was 1.7 MT/ha (Moshi *et al*., 1997). These figures show that only the USA and Europe reported a commercially viable yield, which came about through the use of hybrids together with improved management practices (USDA, 1996). So the question remains what are the future prospects in Africa? Bantayehu (1985) reported that the available technologies are inadequate, while Olson and Sander (1988) said the adoptions of improved management are necessary**.**

Maize has been improved from a wild grass to one of the most productive crops. Its breeding work, especially for hybrid development started in the early 1900s with the work of Shull (1909) and others. Maize breeding has been effective in developing improved varieties and hybrids to meet the rapidly changing cultural and environmental

conditions of this century. The development of the commercial seed industry is in testimony of successful breeding methods that have been followed for the economic production of acceptable high quality hybrid seed (Hallauer and Miranda, 1988).

Agricultural crops get damaged if they are not protected against diseases (Hagenboom, 1993). Crop protection, therefore, is necessary not only for the maintenance of production capacity, but also to prevent negative effects on the quality of the products (Hagenboom, 1993; Ayliffe and Lagudah, 2004; Hall, 2003). For example, fungal diseases are economically important to man because they reduce both crop yield and quality. Consequently, many governments use a lot of their resources to control crop pathogens. Losses in maize yield bring famine, reduce industrial productivities, cause unbalanced diets, malnutrition and even death etc. It is clear that crop losses are very high and every effort should be taken to reduce them. Crop protection may be realized by different means: chemical, biological, plant resistance, molecular techniques, etc. Of these, plant resistance is highly preferable. It is inexpensive as compared to others, best for the farmer, and biologically safe to the environment (Parlevliet, 1993; Gevers and Lake, 1994).

Gray leaf spot (GLS) is one of maize foliar diseases that is currently recognized as the most yield limiting disease of maize world wide (Ward and Nowell, 1998; Dunkle and Levy, 2000; Gordon *et al.*, 2004), especially in the USA and Africa. GLS is caused by the fungus *Cercospora zea maydis.* This disease was first reported in the USA in 1925 (Tehon and Daniels, 1925). The disease has continued to expand its geographic distribution and increased its intensity over the past 25 years (Ward *et al.*, 1999). In addition to the USA and Africa, GLS has been reported in Asia (Coates and White, 1994) and South America (Latterell and Rossi, 1983).

GLS is of major importance in Africa with its resultant severe reductions in grain yield and quality. This disease poses serious repercussions concerning the food security and nutrition of the African countries further widening the food deficits in these nations (Ward *et al.*, 1999). The impact of this disease can be ascribed to the fact that maize is

the major staple food for most of the indigenous rural populations of Africa (CIMMYT, 1990). Estimates of yield losses attributed to GLS for example in South Africa, are frequently as high as 30-60 percent for moderately resistant and susceptible hybrids (Ward *et al.,* 1997). Other deleterious effects of GLS are increased lodging; reduced grain quality, poor silage quality and no mechanical harvesting can be done on the highly infected hybrids (Gevers and Lake; 1994, Ward *et al.*, 1999).

An outbreak of this disease in Kwazulu Natal, South Africa resulted in the first official report from the African continent (Gevers *et al.,* 1994). In Africa, GLS has now become pandemic (Nowell, 1997). Large areas of farmland currently under maize production in other geographical areas are potentially at risk, should the pathogen be introduced and environmental conditions become favourable to this disease. The current rise in the incidence and spread of the GLS has been ascribed to conservation tillage, increase in plant debris from season to season and use of genetically vulnerable maize genotypes (Roane 1950; Ward *et al.,* 1994). Furthermore, the disease rise in many countries has been aggravated by the fact that many national maize breeding programs have released numerous inbreds, hybrids, and germplasm with no direct or conscious selection for resistance to GLS. (Elwinger *et al*., 1990; Huff *et al.,* 1988).

In Tanzania, maize is the most important staple food grown and consumed. Approximately 1.7 million hectares are annually cultivated with maize which accounts for about 60 percent of the total area planted with cereal crops (Moshi *et al.,* 1997). Although grain yields are highest in the highlands of Tanzania, where there is sufficient and reliable rainfall, maize production per unit area is still low. This low production is a result of a variety of constraints including diseases, poor soils, poor weed control, pests (Moshi *et al.,* 1997), etc. Among these production constraints, gray leaf spot disease is the most yield limiting factor in the Southern Highlands of Tanzania (SHT).

Gray leaf spot disease was reported for the first time in Tanzania (SHT) during the 1994/95-rain season (Lyimo and Mohammed, pers. Comm.). But a recent study at Uyole research institute revealed that only one (or at most two) commercial hybrid (UH615) are

resistant to GLS. However, some inbreds such as P62145 and others developed from population 62 (an open pollinated variety) are good sources of GLS resistance with potential to be deployed effectively in the SHT maize breeding program for the production of GLS insensitive maize cultivars as they express different type of resistances to GLS. For example highly resistant inbreds show fleck type of lesions, while moderately resistant display chlorosis, and highly susceptible materials exhibit necrotic spots. This disease has now been reported from other regions in the country and is still moving northward following the major corn belt of Tanzania. Thus, it is possible that if GLS disease is not managed properly it may spread to the rest of the country. Hence the hypothesis of this study was that hybrids in the SHT highly succumbed to GLS disease. GLS management measures are therefore necessary for increased and sustained maize production.

Current control measures of GLS like field sanitation, use of fungicides, crop rotation, combination of methods, etc, are not very effective (Dunkle and Levy, 2000; Ward *et al.*, 1997) and are either uneconomical or environmentally unsound (Ward and Nowell, 1998; Elwinger *et al.,* 1990) or both. Furthermore, the pathogen may develop resistance to the fungicides (Ward *et al.,* 1999). Breeding for disease resistance using conventional methods seem to be somehow limited by the quantitative nature of the inheritance of GLS resistance (Saghai Marroof *et al*., 1996). Thus, the need to integrate conventional breeding strategies (i.e. phenotypic-based selection) with molecular techniques such as marker assisted selection (MAS). These strategies seem to be the most feasible approaches for breeding resistance with quantitatively inherited traits like GLS. Conventional technique is very effective for highly inherited traits while molecular methods are effective for lowly inherited traits. So integrating phenotypic data with molecular information seem to increase the efficacy of selection as they complement/supplementation each other. The advantage of molecular selection is to pyramid GLS resistant quantitative loci in the selection. Also, molecular strategies are able to identify those hybrids which have escaped the disease by chance during the selection process.

In view of the seriousness, destructiveness, the rapid spread, crop losses due to GLS and the importance of maize to the Tanzanians (as the most staple food and or /cash crop), there is thus an urgent need to manage this disease. The crop losses due to gray leaf spot disease stipulated this study. Thus the main objective of the study was to effectively control GLS disease in the studied area by using an efficient, cost effective, more durable, friendly and safe to the environment method of integrating conventional with molecular techniques of maize breeding. Furthermore, this research investigated the effects of genotype x environment on GLS, yield and other agronomically important traits of the studied materials across multi-locations. Also, this study aimed to identify the most suitable locations for the hybrid production and their stability of performance in many environments. Finally, this work aimed to produce commercial GLS resistant hybrids for the farmers in the SHT, to increase farmer's choice of growing GLS resistant hybrids and or in case of GLS resistance break down. In order to achieve the study main goal, it was important to assess the genetic diversity or composition of the SHT breeding materials and then apply this knowledge to meet the research objectives. This study combined conventional with molecular techniques of plant breeding to produce high yielding GLS insensitive hybrids in an efficient manner. The potential benefits of this study is to apply molecular techniques with phenotypic-based selection that are able to increase the efficacy of identifying best line combinations with maximum heterosis, and also improves screening procedures for identifying hybrids with stable GLS genetical resistance across environments. Thus, the main objectives of this study were:

1. To predict best line combinations for the production of commercial GLS resistant hybrids in the SHT. Hence it was necessary to study the genetic diversity of the selected moderately and highly GLS resistant maize inbreds by applying AFLP-DNA fingerprinting protocol.
2. To compare and correlate genetic distance (GD) of the maize lines with important morphological traits of their F_1 progeny.
3. To evaluate hybrids for GLS resistance in multi-environments by using phenotypic–based traits in order to assess their yields, identify their adaptation areas for optimum production and evaluate their stability of performance.

4. To do a preliminary study of developing putative PCR based molecular marker bands linked to GLS resistant genes which will be used in marker-assisted selection with phenotypic selection as a way of increasing the efficacy of selecting GLS resistant hybrids.

Chapter two describes a detailed review of the available literature concerning GLS disease in maize. This chapter has several sections which deal with the discovery and the outbreak of GLS in the world, the biology of the GLS causative fungus *Cercospora zea maydis*, the development GLS epidemiology, symptoms and importance of GLS, the current control methods of GLS and lastly the use of knowledge of DNA molecular markers in maize resistance breeding. The rationale of this review is to generate knowledge, guidance, direction and methodologies, which could assist breeders to develop maize hybrids with durable GLS resistance in different environments by improving the current conventional methodologies and integrating with modern technologies of biotechnology.

In Chapter three, I assayed the genetic diversity of the selected SHT maize germplasm by using the AFLP-DNA fingerprinting protocol (by applying the knowledge of pairwise genetic distance of lines) so as to predict the potential of these inbreds to produce high yielding GLS resistant hybrids. Pairwise genetic distance of lines is based on the theory that genetically dissimilar parents produce high yielding hybrids when crossed. DNA fingerprinting using the AFLP protocol was also important as a way of assessing the existing genetic variation in the selected germplasm and to ensure against depletion of the existing gene pools. This chapter forms the basis of my thesis for marker development as some AFLP bands could be linked to certain genes of interest which might be used as putative markers for GLS-genotype selection purposes. The other objective of AFLP-DNA fingerprinting was to investigate the usefulness and the potential of AFLP data to determining the best line combinations for increased hybrid heterosis. Information from inbred DNA fingerprinting also helps to give information on heterotic groupings which the later assists to give information on general and specific combining ability and possibly prediction of best line combiners.

Chapter four looked at the inbred AFLP-GDs and their F_1 phenotypic trait associations. It also investigated the inheritance of GLS resistance in maize. The information obtained from chapter four could complement data of chapter three required to predict best line combinations for commercial GLS resistant hybrid production. Furthermore, this chapter generated information of correlations between agronomically important traits that can be simultaneously selected for and fixed in inbred lines especially when they are tightly linked during inbreeding programs.

Chapter five focused on production, evaluation and identification of GLS resistant hybrids in multi-environments. The rationale of this chapter was that resistance breeding appears to be superior to other control methods such as use of fungicides, cultural control, and crop rotation. The limitations of the latter control strategies are that they are either expensive or less effective or both. Such demerits prompted the use of resistance breeding as the best GLS control method. It is important to note that information of pairwise GDs of inbred lines that were predicted using AFLP-fingerprinting (chapter three) were then combined with studies of correlations of GDs of inbred lines with morphological data and inheritance of GLS resistance (chapter four) enabled to identify high yielding crosses of maize which had high level of GLS resistance. Furthermore, evaluation of hybrids for GLS resistance was necessary in order to quantify the effects of environment and G x E on the phenotypic expression of different hybrid traits. These hybrids were evaluated in “hot spot” GLS disease pressure areas across locations and seasons in the SHT as a prerequisite for evaluating genotypes for GLS resistance. Other objectives of this chapter were to identify areas of adaptation of these hybrids for their optimum commercial production, to increase the number and choice of GLS resistant hybrids for the farmers in the SHT and finally, aimed to ensure a constant supply of GLS insensitive hybrids in case of GLS resistance breakdown of the commercial hybrids.

In Chapter six, Putative cleaved amplified polymorphic molecular marker bands were developed, which are tightly linked to GLS resistant QTLs in maize. The rationale for developing these putative markers was due to the fact that currently, in Tanzania, to the

best of my knowledge, there are no molecular markers that have been developed in the past /on going work which are linked to the GLS insensitive genes. Thus, this chapter attempted a preliminary study on the development of CAPS markers for future application in MAS strategies in the SHT maize hybrid breeding program. Some of the merits of molecular selection are that it is able to identify those genotypes which have escaped the disease by chance. Also, molecular marker selections help to pyramid GLS resistant factors during the selection process. The importance of PCR-based molecular markers over other DNA markers is that a lot of genotypes can be screened for the gene of interest in a shorter period of time and at a lower cost. The development of markers using the Tanzanian population was significant to advancement in the SHT breeding program, as markers from different backgrounds usually become less effective and reliable when used across other backgrounds.

Finally, it is my believe that combining MAS and phenotypic selection to identify GLS resistant hybrids, as a long term strategy in the SHT breeding work will optimize the efficacy of selection gains for the breeding program when compared with phenotypic or marker selection alone. I anticipate that combining molecular technology with phenotypic selections could complement each other and effectively improve breeding for resistant to GLS disease.

Lastly, this thesis presents the first molecular-genetic study in maize in the SHT. It explored the knowledge and application of the associations between parental GD and F_1 crosses for the identification of best line combinations which could be used to produce commercial GLS resistant hybrids. The impact of this research will directly result in the germplasm genetic improvement as well as release of GLS resistant hybrids.

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CHAPTER 2

LITERATURE REVIEW

Gray leaf spot (GLS) of maize is a foliar disease caused by the fungus *Cercospora zea maydis* (Tehon and Daniels, 1925). The disease was first described from samples taken in Illinois, USA, in 1925. There were a few reports of the disease in the 1940s (Arndt, 1943; Lehman, 1944; Roane, 1950). In 1950s (Roane, 1950) and 1960s (Kingsland, 1963) the disease was relatively rare. However, during the early 1970s the number of outbreaks reported increased (Latterell and Rossi, 1983). It is now a disease of agricultural importance in the warmer regions of USA (Latterell and Rossi, 1983; Bubeck *et al.,* 1993, Saghai Maroof *et al.,* 1996; Wang *et al.,* 1998) and in South Africa (Gevers *et al.*, 1994; Ward *et al.,* 1997).

Significant outbreaks of GLS have also been reported in South America, for example in Peru, Colombia, Brazil, Trinidad (Chupp, 1953). In South Africa, the disease was first observed at Greytown areas during the 1988/89 season and then at Cedara in 1992 (Ward and Nowell, 1997; Gevers *et al.,* 1994). It has since spread throughout the province of Kwazulu-Natal and in neighbouring provinces and countries (Ward and Nowell, 1997). In Tanzania, it was first reported during the 1994/95-rain season (Lyimo and Mohammed pers. Comm.).

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2.1 The genus *Cercospora*

Cercospora Fresenius, is one of the genera of the fungi imperfecti (fungi which contain conidial or imperfecti stage). This genus has relatively broad and large conidia. Mature conidia are 70-180 μm long and 5-6 μm wide at the base. They are tapering to 2-3 μm at the apex (Latterell and Rosi, 1983). As with most fungi, however, these dimensions as well as those of conidiophores vary considerably with the conditions under which these structures are formed especially with respect to natural or artificial substrate, moisture, light and age of stroma. Stromatic tissue of the fungus is normally found in the substomatal chambers of the infected host tissues. These stromatic tissues later produce conidiophores that merge in clusters of up to 20 in number. Conidiophores can be found emerging from leaf blades, sheaths and husks (Latterell and Rosi, 1983). The principle characteristics of this genus include the following parts:

(1) **Conidia:** These are the principle source of the characters, which separate *Cercospora* from other genera, and that differentiates species within the genus itself (Chupp, 1953). Most true species of *Cercospora* have their conidia hyaline or pale coloured (Chupp, 1953). About 6-10 septa and germinate readily (within 3 hours) from any cell, three or four germ tubes called "appresoria" typically emerging during infection process on corn tissues. It should be noted that spermagonia (bodies containing permatia), the presumed male gametes of the sexual state of the pathogen (Latterell and Rossi, 1977) develop in mature lesions from the stromatic cells in each substomatal cavity. They are filiform, obclavate, straight or curved not cylindrical, tips acute to obtuse, multiseptate, and borne singly and terminally or by the further growth of conidiophores.

The **base of the conidia**: The form of the basal end of the conidium of the genus *Cercospora* is probably the most dependable character on which differences in species of *Cercospora* are based (Chupp, 1953). For example if one species is found in any given country/environment with truncate base the same base will differentiate it wherever collections are made.

(2) The **conidiophores**. These are the pathogen structures which arise singly from procumbet heads or in fascicles, from interlacing threads or from a distinct troma. Conidiophores are septate or unicellular less dependable than the conidia in furnishing stable characters of the genus (Chupp, 1953). They, however have a number of specific variations, which should be used to determine the identity of any given specimen. For example colour. The depth and uniformity can be used in any quick identification. Some conidiophores have the same colour throughout the entire length; others are pale near the tip. But most of them are usually black, simple, arising in clusters and bursting out of leaf tissue, bearing conidia successively on new growing tips (Chupp, 1953).

(3) Their **mycelia** are internal or external, filamentous, branched, septate, hyaline or coloured.

(4) **Stroma** are lacking to prominent. When present pale coloured to black, globular, irregular or elongated, mostly borne superficially on the affected tissue or finally become erumpent.

2.2 The African population structure of *Cercospora zea maydis*

In order to determine the African population structure of C*ercospora zea maydis* and its relationship to the species that occur in the USA (Dunkle and Levy, 2000) collected several disease samples and fungal cultures at [CIMMYT] Mexico DF, in Zimbabwe, Uganda, South Africa and Zambia. These samples were analysed using Amplified Fragment Length Polymorphism (AFLP) and restriction of internal transcribed spacer (ITS) regions and 5.8s ribosomal DNA (rDNA). They were also morphological and culturally characterised. Results of these analyses revealed that; (a) The ITS and rDNA regions were monomorphic, further indicating genetic homology and very similar haplotypes among the African isolates; (b) genetic diversity within the African population was limited. The average within population similarity was 97.6 percent; (c) the primary taxonomic characters of the African isolates were similar to those of the

isolates from the United States of America; (d) isolates of Africa and the group II isolates of the USA from the eastern USA did not produce detectable quantities of Cercosporin crystal (a photo-activated phytotoxin produced by many species of *Cercospora* (Daub, 1982) over a period of 3 weeks and both grew at a substantially slower rate than isolates of *Cercospora zea maydis* group I; (e) based on morphological characteristics and molecular-genetics criteria, the African population of *Cercospora zea maydis* is equivalent to the population of *Cercospora zea maydis* designated group II. These results show that the African population of *Cercospora zea maydis* has probably a limited or no genetic diversity.

Studies using AFLPs and the ITS region on genetic variability of USA isolates (Dunkle and Levy, 2000; Wang *et al.,* 1998) showed that the pathogen population consists of two taxonomically indistinct but genetically distinct sibling species that are partially sympatric, and thus, are considered sibling species. A genetic distance of approximately 80 percent separated these groups from each other, and within group similarity were approximately 93 (group I) and 94 percent (group II) respectively. They also reported that the mechanisms by which genetic diversity might arise in *Cercospora zea maydis* are unknown. Para sexuality has never been demonstrated in this species and the involvement of the sexual stage in the life is debatable. Latterell and Rossi (1977) observed spermagonia bearing spermatia which are considered as sexual reproductive structures in some cultures. But their role, if any, is unknown (Dunkle and Levy, 2000). However, other investigators (Beckerman and Payne, 1982) did not observe these putative sexual reproductive structures.

2.3 Variability of virulence of *Cercospora zea maydis*

There is little evidence that the virulence of *Cercospora zea maydis* is changing (Thompson, 1987; Wang *et al.,* 1998; Dunkle and Levy, 2000) or that races of the pathogen exist. However, Latterell and Rossi (1974) described variation in certain structural, cultural and metabolic characteristic among the isolates of *Cercospora zea maydis* and concluded that such variation also may be expressed in pathogenecity or

aggressiveness. However, Bair and Ayers (1986) reported variation in components of parasitic fitness (disease deficiency and virulence measured as lesion length). Dunkle and Levy (2000) reported that isolates within both groups I and II of the USA exhibit a range of aggressiveness when inoculated on maize hybrids but disease symptoms were identical and no significant differences were noted in the severity of the disease incited by both groups. They concluded that the substantial genetic difference between the two groups in the USA was not expressed as differential virulence. While Wang *et al.* (1998) showed that the relative disease severity ratings of hybrids with a range of phenotypes did not change significantly from location to location. They also observed neither of the siblings of *Cercospora* species had selected maize genotypes which are more susceptible to one than.

Also note that in field trials, significant G x E (hybrid x location) interaction is frequently observed (Bair and Ayers, 1986; Bubeck *et al.,* 1993; Carson, 1997; Huff *et al.,* 1988 and Thompson, 1987). Such variation may be due to differential sensitivities of maize genotypes to environmental factors and thus are predisposed to more severe infection by GLS development. Such sensitivities usually have an impact on disease severity, since the QTL effects associated with resistance to GLS are inconsistent over environment (Bubeck *et al.,* 1993; Wang *et al.,* 1998) resulting from genotype x environmental interaction, random variation within environment or in some cases false positives (Bubeck *et al.,* 1993). Although numerous instances of G x E interactions have been detected by classical quantitative genetics analysis, recent results from RFLP mapping tentatively suggest very little environment by QTLS interaction (Tanksley, 1993).

2.4 Variability of *Cercospora zea maydis* in East Africa

Phyllogenetic analysis using AFLP on the East African population structure of *Cercospora zea maydis* (Okori *et al*., 2003) revealed two major clusters. One large cluster comprised 75 % African and USA group II isolates and the second cluster had four USA group I. Similar grouping was observed with RFLP data. Analysis of

molecular variation (AMOVA) based on AFLP data revealed a significant population structure between American and African populations (ϕF_{st} = 0.07). No population structure was detected among African isolates (ϕF_{st} = 0.01). But a strong and significant structure was obtained between the two pathotypes (ϕF_{st} = 0.19). The AMOVA using RFLP data showed absence of population structure among African populations (ϕF_{st} = 0.01). The Okori *et al*. (2003) study reported that gene flow among African populations was high 0.45. These findings suggest that group II pathotype is predominant in East Africa and gene flow appears to be fundamental evolutionary force accounting for the current genetic structure.

2.5 Disease epidemiology and development

GLS disease development is favoured by extended periods of overcast days, warm temperatures and high relative humidity (Beckman and Payne, 1982; Rupe *et al.,* 1982). High relative humidity, suitable air temperatures, host susceptibility and the presence of a source of inoculum are the conditions necessary to cause a GLS epiphytotic. The absolute rate of change of GLS with respect to time is often best described by the logistic model in which the absolute rate of disease increase dy/dt is a function of ry (1-y) (Jenco 1995; Nutter and Jenco, 1992; Nutter *et al.,* 1994; Ward *et al.,* 1999). Logits = $\log_e$ (p/1-p) i.e. the natural logarithm of the odds corresponding to the probability p of a specified outcome given the existence of a stated attribute (international dictionary of medicine and biology volume 3 in John Willey and sons 1986) and is conveniently expressed as the difference of the two logits. Thus, the rate of disease development of GLS epidemics is driven by three factors that interact in time and space: (1) the initial amount of inoculum (y); (2) the rate of within season pathogen reproduction (r); and (3) the proportion of healthy tissue remaining to be infected (1-y). It follows that the higher the initial level of inoculum the faster the GLS development will occur with respect to time.

Several studies have shown that environmental factors have tremendous impact on the rate of within season gray leaf spot disease development. In Ohio, deNazareno *et al.*

(1992) reported that the rate of GLS progress (r) ranged from 0.13 to 0.17 logits per day (favourable) for disease development and 0.02 to 0.06 logits per day (less favourable) for disease development. In Iowa, Nutter and Stromberg (1999) obtained some what higher estimates of disease increase with rates of disease development ranging from 0.07 in 1991 (moderate favourable) to 0.28 logits per day in 1992 (extremely favourable). In South Africa, Ward *et al.* (1999) reported apparent infection rates of up to 0.16 logits per day during 1991/92-rain season (highly favourable) and up to 0.10 logits per day (moderately favourable). It was suggested that higher apparent infection rates in moderately favourable seasons coupled with longer growing seasons help to explain as to why disease severities are generally higher by the end of the growing season in Africa as compared with the United States (Ward *et al*., 1999; Nutter and Stromberg, 1999).

Studies of the spread of *Cercospora zea maydis* from infected maize debris in the East African soil surface and its progress over time showed that the amount of infected residue on the soil surface in the maize planting was highly significant (Asea *et al*., 2002). The distance from the infected residue significantly affected the foliar disease but the direction from the residue inoculum source did not nor was the distance by direction interaction significant. However, interactions between amount of residue and distance were significant. The percent leaf area affected by GLS, the area under disease progress curve and disease intercepts decreased with distance.

2.6 Symptoms of GLS on maize plants

Symptoms of GLS on susceptible maize are grey to tan linear rectangular lesions delimited by major veins on the maize leaves. Lesions are completely opaque when viewed through transmitted light on leaf, sheath and husk tissue (Smith and White, 1987; Latterell and Rossi, 1983). This is a characteristic of *Cercospora* infection on graminaceous hosts (Latterell and Rossi, 1983). Within 2 weeks such lesions gradually elongate appearing as streaks before developing their dark grayish brown or tan rectangular shape. The lesions remain tan until dense sporulating under humid conditions and then produce a greyish cast, hence the common name. Lesions develop

slowly as compared to other foliar pathogens and are pin- point in size surrounded by a "yellow halo" (Latterell and Rossi, 1983). They require about 2-3 weeks for full expansion. Mature lesions are 1-10 cm long and 2-5 mm wide (Ayers *et al.,* 1984; Latterell and Rossi, 1983; Stromberg, 1986). If adjacent interveinal areas are infected, two single lesions may coalesce to form one broad one. Lesion development usually begins on the lower leaves producing conidia that serve as inoculum for the upper leaves. As many lesions form they coalesce and become more difficult to distinguish and blighting results until all leaves are killed (Stromberg, 1986). Gray leaf spot resistant genotypes display fleck type lesions (Ayers *et al.,* 1984; Latterell and Rossi, 1983), while moderate resistance materials exhibit chlorotic lesions (Roane *et al.,* 1974) and susceptible plants show necrotic spots (Ayers *et al.,* 1984; Huff *et al.,* 1988; Latterell and Rossi, 1983; Saghai Maroof *et al.,* 1993).

2.7 Effects of GLS on maize crop

Losses associated with gray leaf spot occur when photosynthetic leaves are rendered non-functional due to lesions and/or the blighting of the leaves. The blighting and premature death of leaves severely limit radiation interception as well as the production and translocation of photosynthates to developing kernels (Ward *et al.,* 1999). Leaves of susceptible genotypes may become severely blighted or killed as early as 30 days prior to physiological maturity (Jenco, 1995; Ward, 1996).

Gray leaf spot has resulted significant losses in income to grain producers and adversely affects yield and quality of silage maize (Ward and Nowell, 1994; Donahue *et al.,* 1991). This is especially true for the upper eight to nine leaves that contribute 75 to 90% of the photosynthates for grainfill (Allison and Watson, 1996). The components of yield which are most affected by GLS are (a) number of kernels per ear and (b) kernel size. Thus the little food manufactured is diverted from roots and stalks in greater amounts than normal at the expense of the ear, which is the major sink at that time. Ears are incompletely filled with the photosynthates from the leaves and mature prematurely Donahue *et al.,* 1991). Consequently stalk deterioration and lodging and breakages occur (Latterell and

Rossi, 1983; Shurteff and Perdesen, 1991; Ayers *et al.,* 1984; Stromberg and Donahue, 1986). This can also lead to plant death (Donahue *et al.,* 1991).

Percent yield loss is defined as the difference between the attainable yield and actual yield/attainable yield x 100 (Nutter *et al.,* 1993). Using this definition to quantify the yield losses caused by GLS, different loss estimates have been reported. Donahue *et al.* (1991), Ayers *et al.* (1984) and Beckman *et al.* (1981) reported loss due to GLS to be 10-25 percent of the yield potential in endemic areas but can be much higher. Gevers *et al.* (1994) observed up to 40 percent whereas Ward and Nowell (1994) and Ward *et al.* (1997) indicated yield losses up to 50-65 percent with reduction in both yield and quality of silage. Latterell and Rossi (1983) reported the loss due to GLS approaching 80 to 100 percent in epiphytotics. No mechanical harvesting of maize can be done on GLS infected fields. From literature it is clear that GLS causes enormous maize crop losses in both endemic and epidemic situations. This problem has posed many scientists around the world to embark on different research programs looking for solutions to the problem (Ward and Nowell, 1994, 1998; Bubeck *et al.,* 1993). Some of GLS control methods documented include the following:

2.8 Control of GLS disease

2.8.1 Elimination of the corn debris and avoid no till and other conservation practices

Debris serves as good over wintering source of inoculum for the GLS (deNazareno *et al.,* 1992; Ureta, 1985; Payne and Waldron, 1983). It is the fungus within the infected debris from the previous crop that produced conidia following long periods of warm humid weather. Disease levels increase with the amount of residues on the soil surface (deNazareno *et al.,* 1992). Ward *et al.* (1997) used four treatments e.g. no till, chisel, chisel and disc and conventional ploughing leaving stubble residues of 3, 26, 41 and 82 percent on the soil surface. Their results showed that the effective tillage practices of GLS development depended on the amount of stubble remaining on the surface and the

prevailing weather conditions following tillage treatments. These results also revealed that the safest GLS control option is to avoid no till and other conservation practices especially when maize follows maize (deNazareno *et al*., 1992) and that the weather pattern has the greatest effect on the disease development (Payne and Waldron, 1983).

2.8.2 Crop rotation

This cultural practice has been emphasized by several studies (Smith and White, 1987; Ward and Nowell, 1994; Thorson, 1989; Latterell and Rossi, 1983). They said avoiding the inoculum by rotation to another crop would reduce the level of inoculum in the soil. Thus crop rotation can be a promising an alternative control method for two reasons; (a) the pathogen does not survive in diseased maize debris beyond one year (Latterell and Rossi, 1983), and (b) *Cercospora zea maydis* is pathogenic only to maize among crop species (Latterell and Rossi, 1983). However, (Gevers *et al.,* 1994; Gevers and Lake, 1994) reported that discontinuation of conservation tillage is not a viable control option and crop rotation may not be an effective control method.

2.8.3 Use of chemicals

Thorson (1989) reported that fungicides have been observed to reduce GLS severity in the USA, but the application of fungicides is probably uneconomical except for seed producers (Ward *et al.,* 1999). The objective of fungicides is to delay the onset and the rate of development of the disease until the crop reaches physiological maturity. Fungicide application was most effective when spraying commenced as disease severity levels reached 2 to 3 percent of the leaf area blighted, and when lesions were restricted to the basal five leaves of the maize plant. The number of spray application will therefore be a function of the stage of growth of the maize crop when it is initially infected by the pathogen (Ward *et al.,* 1997). With early infections, more fungicide treatments were necessary to provide protection to physiological maturity. Infections, which may occur from shortly before or soon after tasselling, two fungicide applications may be necessary to delay disease development (Ward and Nowell, 1994).

Approximately 3-4 weeks after tasselling, a single fungicide application should be sufficient (Ward *et al.,* 1994). These results indicate that fungicides are highly effective in controlling the development of GLS. Examples of fungicides are carbendazim, flutriafol, difenoconazole, benlate, etc. Although foliar applications of fungicides are effective control but the pathogen may develop resistance to the fungicides and may not be rewarding to grain producers (Ward *et al.,* 1999).

2.8.4 Combination of methods

This refers to the use of fungicides and less susceptible cultivars. Lless susceptible hybrids to GLS disease have been identified (Ward *et al.,* 1994; Ward and Nowell, 1998). However, resistance of less susceptible hybrids may not be adequate to minimize significant yield loss under high disease pressure. A fewer of these hybrids with low susceptibility are high yielding and should be selected for areas subject to heavy disease pressure (Ward and Nowell, 1994). In many cases these hybrids may need timous application of fungicides (Ward *et al.,* 1994). In fact, this is one type of integrated pest management.

2.8.5 Use of GLS resistant cultivars

The adverse economical and ecological effects of mouldboard tillage, use of fungicides and other methods made genetic resistance the best control strategy for GLS disease (Ward *et al.,* 1999; Lipps and Pratt, 1989; Hohls *et al.,* 1995; Saghai Maroof *et al.,* 1996; Coates and White, 1994). Despite the fact that there are several GLS resistant germplasm that have been identified (Ayers *et al.,* 1984; Thompson *et al.,* 1987; Dunkle and Levy, 2000) a high level of resistance has not been incorporated into a commercial hybrid (Ward and Nowell, 1994; Ward *et al.,* 1997; Dunkle and Levy, 2000). But there are increasing number of hybrids, which are less susceptible to GLS (Stromberg and Donahue, 1986; Gevers *et al.,* 1994; Ward *et al.,* 1997). Genetic resistance for the control of GLS is highly effective, easy to use and cost effective method and will play a major role in providing a long term solution to the problem (Lipps and Pratt, 1989;

Elwinger *et al.,* 1990; Gevers and Lake, 1994; Ward and Nowell, 1998; Bubeck *et al.,* 1993). The quantitative nature of the inheritance of GLS resistance was determined by Manh (1977) based on GLS disease rating in F_2 and F_3 generations from a cross between a resistant line (Va14) and a susceptible line (H98). He concluded that additive genetic effects account for 82-96 percent of total variability among generations, with a narrow sense heritability of 31 percent. Other investigators (Ayers *et al.,* 1984; Donahue *et al.,* 1991; Huff *et al.,* 1988; Ulrich *et al.,* 1990; Freppon *et al.,* 1994) reported similar results. While Gevers and Lake, 1994, Elwinger *et al.,* 1990 and Bubeck *et al.,* 1993) added that some genes exhibited major gene with dominant effect. They also identified some outstanding GLS resistant hybrids of 0.46 for the cross of K054W x R055W. Both the GCA (i.e. the general performance of a line in hybrid combinations) and SCA (i.e. best line x line performance) were also highly significant. They noted that R0465W, S081W and R0558W had high GCA indicating greater additivity in some lines. The importance of the additive component of variance suggests that active selection for resistance to GLS in these materials as well as use of the most resistant lines in crosses or in back cross programs should be very successful.

But it should be known that breeding maize for GLS resistance has been limited by (1) quantitative nature of the inheritance of GLS (Ayers *et al.,* 1984; Saghai *et al.,* 1996) and (2) limitation of selection under less than optimum disease pressure (Payne and Waldron, 1983). So the presence of heavy disease pressure is an essential prerequisite to evaluate the levels of GLS resistance (Saghai Maroof *et al.,* 1996).

2.8.6 Use of molecular techniques

Identification of genes controlling traits is critical in order to determine the effect of these genes or quantitative trait loci (QTLs), to study the molecular mechanism of individual genes and to facilitate the transfer of desirable traits in marker assisted breeding programs. Quantitative identification across populations should allow opportunities for marker assisted selection (Bubeck *et al.,* 1993; Lehmensiek *et al*., 2001: Gordon *et al.,* 2004) and pyramiding of genes from different backgrounds into one

line (Bubeck *et al.*, 1993).

2.9 Early genetic studies for GLS resistance

Early studies of temperate adapted germplasm addressed the genetic basis of resistance to GLS by diallel and generation mean analyses and concluded that the resistance is under additive genetic control with some dominance effects (Thompson *et al*., 1987; Ulrich *et al*., 1990; Gevers *et al*., 1994; Coates and White, 1998). Genotypes were selected in these studies resulting in a fixed model (Griffing, 1956). The conclusions reached were therefore applied only for the selected inbred lines. Gevers and Lake (1994) suggested that a single gene in South African germplasm conferred resistance to GLS, but their results have not been confirmed (Gordon *et al*., 2004).

2.10 QTLs identification for GLS resistance studies

QTL mapping studies have made limited progress in identifying consensus QTL for resistance to *Cercospora zea maydis* (Bubeck *et al*., 1993; Saghai Maroof *et al*., 1996; Clement *et al*., 2000; Lehmensiek *et al*., 2001; Gordon *et al*., 2004). Bubeck *et al*. (1993) used two different inbreds, NC250A and DENT, as source of resistance to GLS in the $F_{2:3}$ mapping populations. They identified QTL on five different chromosomes but only one of these, on the short arm of the chromosome 2 was consistent over three environments. Saghai Maroof *et al*. (1996) employed selective genotyping to identify three QTL on chromosome 1, 4 and 8 that collectively explained 44-46 percent of the variation across two generations F_2, and $F_{2:3}$, across two seasons in one location. In test for epistatic interactions, they demonstrated that the QTLs on chromosome 4 had little or no effect when the QTL on chromosome 1 was homozygous**.** In addition, the QTL on chromosome 8 displayed recessive gene action. Using the inbred 061 as a resistance source, Clement *et al*. (2000) evaluated a BC_1S_1 population for 2 year at one site and 1 year at a second site. They found five QTLs all from the resistant parent, which were significantly associated with GLS resistance in both years and locations. Lehmensiek *et al*. (2001) used bulked segregant analysis to identify QTLs on chromosome 2, 3 and 5

associated with resistance to GLS in an F_2 population derived from proprietary parental lines. These four studies collectively utilized five resistant inbreds and chromosome 1, bin 1.05/1.06 is nearest to a consensus QTLs identified, with three of the five inbreds contributing GLS resistance from this region. Gordon *et al*. (2004) reported several markers on chromosome 2L (long arm) and 4L were deemed to be significantly associated with GLS resistance in maize. They documented that such inbred line like V061Y is a good source of resistance to be deployed effectively in USA and South Africa and that intervals on chromosome 2L and 4L were detected in all tests and can be considered them to be suitable QTLs for MAS.

2.11 Linkages between GLS resistance QTL and other disease resistance genes

Quantitative resistance to several maize diseases has been reported (Saghai Maroof *et al.,* 1996). The separation of quantitative resistance into a few QTL enabled to examine their relationship to previously identified maize genes conferring qualitative resistance to other diseases. The chromosomal location of a *Cochliobulus carbonum* race-1 gene that has been previously cloned by transposon tagging (Johal and Briggs, 1992) is also located on chromosome 1 (QTL1) which had largest effect on GLS resistance. *QTL*8 was also found to be closely linked to two *Helminthosporium turcicum* resistant genes, *Ht*2 (Zaitlin *et al.,* 1992) and *hm*1 (Simcor and Bennetzen, 1993). These three genes appear to belong to a resistance gene cluster since neither *Ht*2 nor *Hm*1 *per se* contribute to GLS resistance. Significant effects of RFLP markers on resistance to *Gibberella zea* were found on chromosome 1, 2, 4, 5, and 10 (Pe *et al.,* 1993). Freymark *et al.,* (1993) reported that chromosome 2, 4 and 8 each had at least one marker with some resistance to *Helminthosporium turcicum* at $\propto = 0.05$ level of significance.

2.12 Molecular markers

Molecular markers should not be considered as normal genes as they usually do not have any biological effect and instead can be thought as constant ‘land marks’ in the genome.

They are identifiable DNA sequences (Russell and Thompson, 1989) that were found at specific locations of the genome and transmitted by standard laws of inheritance from one generation to the next. They relied on DNA assays in contrast to morphological markers and biochemical markers. Many molecular markers have been identified in the intergenic DNA within the introns of the genes; a few have been recorded within the recording sequences (exons) of the genes (Tom and Read, 1999).

2.13 Types of DNA markers

Different types of molecular markers exist. The principles, potential power, advantages/ disadvantages and their application in different area of science can be reviewed (Dogson *et al.,* 1997; Rafalki, 2002; Buitkamp and Epplen, 1996; Botha and Venter, 2000). There are two classes of DNA markers. Those based on DNA-DNA hybridisation and those on the polymerase chain reaction (PCR) amplification of genomic sequences (Cobb and Clarkson, 1994; Botha and Venter, 2000; Liu and Cordes, 2004).

2.13.1 Amplified fragment length polymorphism (AFLP)

The amplified fragment length polymorphism technique was developed by Zabeau and Vos (1993). An AFLP is a highly reproducible multiplex assay and has the ability to produce large number of polymorphic loci (Myburg *et al.,* 2001; Botha and Venter, 2000). They are used extensively for high resolution genetic linkage mapping (Blears *et al.,* 1998), fingerprinting in plants, analyse genetic diversity in many species (Mackill *et al*., 1996; Maughan *et al*., 1996). Furthermore, the AFLP is a robust and rapid technique for analysing many genetic loci and can be used to facilitate breeding programs based on combining and selecting new combinations of genotypes to maximize the rate of line improvement (Liu and Cordes, 2004).

This technique involves three steps, namely (1) restriction digestion of genomic DNA and ligation of oligonucleotide adaptors; (2) preselection and selection amplification of

restriction fragments; and (3) gel analysis of amplified fragments (Vos *et al.,* 1995). The fragment sizes range from 60 to 1500 base pair (50 to 100 fragments are co-amplified per AFLP cycle (AFLP Perkin-Elmer, 1996). The amplified fragments are radioactively or fluorescently labelled and separated on sequencing gels. Results can be silver stained to save on costs. PCR amplification of restriction fragments is achieved by using the ligated adapters on to restriction ends as target sites for primer annealing. The preselection and selection is achieved by using primers that extend into the restriction fragments amplifying only those fragments in which the primer extensions match the nucleotide flanking the restriction sites (Vos *et al.,* 1995). Like RAPDs, AFLP markers are inherited as dominant markers although software packages are now available (AFLP QuantaPro, Keygene) for codominant scoring of AFLP bands (Liu and Cordes, 2004). Codominant scoring is also possible if PCR markers are designed by cutting individual bands of interest from the AFLP gel sequence and design specific primers for each band. This results in one or more codominant markers that can be scored using traditional analyses (Liu *et al.,* 1999).

2.13.2 Sequence characterised amplified region (SCAR)

Problems that are associated with RAPD analysis have necessitated scientists (Demeke and Adams, 1994; Xu and Bakalinsky, 1996) to develop a new marker system that can be used in routine screening. The SCARs also known as sequence tagged sites (STS) or allele specific associated primers (ASAP) (Kelly, 1995; Schachermayr, *et al.,* 1994; Gu *et al.,* 1995 and Olson *et al.,* 1989). A SCAR is a genomic DNA fragment at a single genetically defined locus that is identifiable by PCR amplification using a pair of specific primers (Paran and Michelmore, 1993).A SCAR differs from RAPDs in that it makes use of a set of specific oligonucleotides that amplify a specific region of the genome, usually of low copy number (Weedern *et al.,* 1994; Ohmori *et al.,* 1996; Talbert *et al.*, 1994). When designing a SCAR primer, the RAPD fragment linked to the gene of interest is cloned and then sequenced. The first 10 to 15 bases, together with the RAPD primer sequence, are used to design a more robust primer that results in a less complex banding pattern than the original RAPD primer (Procunier *et al.,* 1997;

Schachermayr *et al*., 1994; Ohmori *et al*., 1996; Dedryver *et al*., 1996). It is also possible to design primers from internal sequences where there are point mutations, deletions or insertions (Feuillet *et al*., 1995).

The advantages of SCARs over RAPDs are that they are more reproducible and show less variability between labs (Schachermayr *et al.,* 1994; Paran and Michelmore, 1994; Melotto *et al.,* 1996), can be used as markers and mapped onto a genetic map as physical landmarks (Paran and Michelmore, 1993). Can be used in position cloning of genes (Salentjin *et al*., 1995) etc. One of the limitations of SCARs is the need to sequence before the primer can be designed whereby more time is needed in cloning the fragment (Talbert *et al.,* 1994). Conversion of a RAPD and a RFLP marker to a SCAR marker can be tedious and difficult (Botha and Venter, 2000; Blake *et al.,* 1996). Tsumura *et al.,* 1997 and McDernott *et al.,* 1994 reported that SCARs can amplify more than one copy of an allele and not all RAPD markers can be converted to SCAR markers (Paran and Michelmore, 1993; Botha and Venter, 2000).

2.13.3 Microsatellite or simple sequence repeats (SSR)

Although some RFLPs as well as many RAPD markers recognize more than one locus in a given genome, the number of loci recognized is limited as is their polymophic content (Winter and Kahl, 1995). Markers derived from small, tandemly arranged repetitive elements overcome this limitation (Winter and Kahl, 1995). Such markers are called microsatellite or minisatellite or simple tandem repeats (STR) or simple sequence repeats (SSR) because their sequence organisation resembles the tandemly arrangement of classical satellite DNA. These sequences are tandemly dispersed throughout the eukaryotic genomes and are often highly polymorphic due to variation in the number of repeats (Litt and Luty, 1989; Tautz, 1989). SSR have been found inside gene coding regions, introns and in the non-coding gene sequences (Liu *et al.,* 2001). Tandemly repeated basic motifs of 2 to 6 base pair (Saiki *et al*., 1988) such as (GA)n, (GT)n, (TG)n or (AAT)n are very common for SSRs. The SSR loci can be amplified by the PCR using primers, which are complementary to the region flanking the repeats.

Microsatellites are highly polymorphic and provide codominant markers with Mendelian inheritance (Beckman and Soller, 1990). These markers are able to detect a much higher level of genetic variation than any other class of markers and are crucial for genetic analysis of organisms with narrow genetic base (Kam-morgan *et al*., 1989).

In plants it has been demonstrated that SSRs are highly informative locus specific markers in many species (Taramingo and Tingey, 1996; Wu and Tanksley, 1993). Other uses of SSRs include linkage analysis (Lothrop *et al.,* 1989; Wells *et al*., 1989), identification of species and cultivars (Weising *et al*., 1998; 1991a), marker assisted breeding (Beckman and Soller, 1990) etc. Due to their varying repeat numbers at a given locus the elements frequently change their length by slippage mispairing during replication and other less understandable processes (Jeffreys *et al*., 1988; Zischeler *et al*., 1992). The surrounding single copy sequences are normally not affected and therefore provide a variable source of polymorphisms for many purposes. One advantage of microsatellite is that primers developed for a particular species can be applicable across a wide range of taxa (Moore *et al*., 1995; Schloetter *et al*., 1991). They seem to provide readily detectable markers for agronomically important genes and quantitatively inherited traits and can facilitate their handling in segregating population (Rodel *et al*., 1998).

2.14 Application of molecular marker technology

Molecular markers provide a remarkable improvement in the efficiency and sophistication of plant breeding and are currently the most important application of molecular biology to plant breeding (Langridge and Chalmers, 1998). Molecular markers allow the construction of high-density linkage maps (Jones *et al.,* 2003; Mitchel *et al.,* 1997, Diwan *et al.,* 2000). Fine mapping of QTLs has sometimes revealed the presence of tightly linked loci affecting the same trait (Fridman *et al.,* 2002; Steinmetz *et al.,* 2002). Molecular markers can be used to test and select traits without performing length field trials. Most importantly molecular markers play greater role in identifying different genes responsible for desirable traits. One method of using molecular markers

for selecting desirable genes is in marker-assisted selection breeding programs. Usually breeders use coupling phase markers (markers only present in resistant plants) to select for plant traits in backcrossing procedures (Kelly, 1995). Haley *et al.* (1994a) demonstrated that the efficacy of MAS could be improved dramatically by selecting against a repulsion phase markers (markers linked to coupling phase to the susceptibility allele). Molecular markers therefore provide an important genetic tool where traditional studies have been difficult (Keim *et al.,* 1990). Markers facilitate establishing genetic linkage among markers and linkages between markers and QTLs.

2.15 The success of MAS

The success of MAS is influenced by relationship between the markers and the genes of interest (Dekkers, 2003; Blears *et al*., 1998) as follows:

(a) Gene assisted selection (GAS). In this case molecular markers are located within the gene of interest (Fridman *et al.,* 2002; Steinmetz *et al.,* 2002). On the other hand it is most difficult to find these kinds of markers.

(b) The marker is in Linkage disequilibrium (LD). LD is the tendency of certain combinations of alleles (e.g. M_1 and Q_1) to be inherited together (Morgante and Salamini, 2003; Weir, 1996; Thornberry *et al.,* 2001). Population wide LD can be found when markers and genes of interest are physically very close to each other and or when lines or breeds have been crossed in recent generation. Selection using these markers can be called LD-MAS. So additional work is needed to determine the extent of LD in crop species and to provide guidelines on the marker density needed to reveal significant associations. (c) The marker is in a linkage Equilibrium (LE). Selection using these markers can be called LE-MAS. This is the most difficult situation of applying MAS and that's why there is an argument that if Fisher's infinitesimal model (many genes with small effects) applies, the efficiency of MAS will always be inferior to that of phenotypic selection even if all genes are known and mapped (Bernardo, 2001).

2.16 Limitations in utilising marker loci-QTLs associations

A current problem in the use of molecular markers is that they are often only effective in the genetic backgrounds in which they were developed. When screening other backgrounds they are less effective and reliable. Routine testing is thus needed to determine the efficacy of markers in other genetic backgrounds (A-M Botha pers. comm.). Others include sample size. The larger the sample size the higher the proportion of additive genetic variance likely to be detected by markers. Markers are effectively used when the linkage between the marker and QTL are tightly linked especially in GAS (Fridman *et al.,* 2002; Steinmetz *et al.,* 2002) and in LD (Weir, 1996; Thornberry *et al.,* 2001; Morgante and Salamini, 2003).

2.17 Marker loci-QTLs associations for GLS resistance in maize

One method of controlling GLS is to develop hybrids with genetic resistance (Dunkle and Levy, 2000; Coates and White, 1998; Gevers and Lake, 1994; Bubeck *et al*., 1993). A number of recent investigations utilized isozyme, allozyme, and or RFLP/RAPD markers to identify and characterize gene action of QTL (Beavis *et al.,* 1991; Keim *et al.,* 1990; Beamount *et al.,* 1996; Saghai Maroof *et al.,* 1996). The QTL were identified on the basis of marker associations with GLS means over all ratings taken over environments (Bubeck *et al.,* 1993). Results showed that individual markers accounted for 4 to 26 percent of the phenotypic variation. Quantitative trait loci associated with GLS were inconsistent over environment (Bubeck *et al.,* 1993; Wang *et al.,* 1998). Bubeck *et al.* (1993) observed that one region on chromosome 2 was associated with GLS resistance in three populations. Additive genetic variance was displayed for nearly all markers associated with GLS resistance. Heterozygosity calculated as percentage of heterozygous loci over all RFLP markers or percentages of marker loci heterozygous in individual plants and mean trait value was not significantly correlated with GLS ratings**.**

2.18 Correlations between mean GLS scores over environments

Pairwise comparisons (Bubeck *et al.,* 1993) of any two subsequent GLS ratings resulted in positive correlations ($p \leq 0.05$) ranging from 0.17 to 0.82 for 3 populations. As the number of days between ratings increased the correlations tended to decrease suggesting that different genes could be involved in early season resistance versus late season resistance. Gray leaf spot often does not begin to develop until several days after flowering and in general late maturing lines tend to be more resistant than early lines (Bubeck *et al.,* 1993). Saghai Maroof *et al.,* (1996) and Bubeck *et al.,* (1993) did preliminary investigations of QTL for number of days after planting to silking (DAPS) to determine whether any regions were similar to those for GLS resistance. Results showed that the region on chromosome 4 (markers P10200597, P10200595, BNL7.65 and UMC15) were associated with both DAPS and GLS entry means over environments. In addition, markers on chromosome 2, 3, and 8 were significantly associated with DAPS in one location and were associated with GLS means in the same location in previous year. Therefore some QTLs for DAPS and GLS means do appear to correspond, resulting from pleiotropic effect or linkage of genes controlling the two traits.

In summary, from literature review, it is revealed that GLS of corn is not yet completely solved in many parts of the world including Tanzania (Lyimo per. comm.). GLS disease is still causing enormous maize yield losses in both endemic and epidemic conditions (Ward *et al.,* 1997; Dunkle and Levy, 2000). This situation is worse in those countries that use maize as a main staple food or as an export crop. Such losses bring famine, food shortages, etc which are always aggravated by bad weather**.** The current control measures of GLS are not very effective and are either uneconomical or environmentally unsound or both. Breeding for disease resistance by using the normal conventional methods seem to be somehow limited by the quantitative nature of inheritance of the GLS resistance (Bubeck *et al.,* 1993; Gevers *et al*., 1994; Pratt *et al*., 2003). It would therefore be wise to consider both phenotypic and molecular (MAS) as complementary breeding tools for efficient resistance breeding to GLS.

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CHAPTER 3

THE PREDICTION OF BEST LINE COMBINERS AND HETEROSIS IN TANZANIAN MAIZE BREEDING LINES THROUGH THE USE OF AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLPs)

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ABSTRACT

Amplified fragment length polymorphism DNA markers have been used to assist plant breeders in the choice of maize parents for commercial hybrid production. However, maize yield in Tanzania is significantly reduced by gray leaf spot (GLS) disease, which is now regarded as the most yield limiting disease of maize worldwide. Thus combining GLS resistance genes and high yielding traits in hybrids is an important breeding strategy. The main aim of this study was to assess the genetic diversity of Tanzanian germplasm and to predict the potential of these inbreds in producing high yielding GLS resistant hybrids. Furthermore, the potential of such data to predict the best line combiners was investigated. This research used AFLP-DNA fingerprinting data from 21 moderately and highly GLS resistant maize inbreds. Results revealed that the genetic distance (GD) of some intergroup crosses were as high as 0.5. Theoretically, these intergroup hybrids with high GD could potentially produce high yielding GLS resistant hybrids. However, such hybrids would require field-testing in order to confirm these observations. Finally the results revealed high f values consistent with other reports in maize. In summary these results also corroborates the usefulness of AFLP in genetic diversity studies of germplasm, prediction of best line combiners and high heterosis level for commercial maize hybrid production.

Keywords: Coancentry coefficient (f), genetic distance, inbreds, interpopulation, intrapopulation,

Introduction

Development of inbred lines, followed by their assignment into appropriate heterotic groups and planning the desired crosses for superior hybrid production requires adequate knowledge of genetic diversity (Hallauer *et al.,* 1988). This applies particularly to maize hybrid breeding where recognition and exploitation of heterotic patterns between genetically dissimilar sources of germplasm are important for success and also when it comes to proprietary issues and plant variety protection. Genetic diversity in cereal collections is critical in finding new alleles that will improve yields to fight world hunger (Warburton *et al.,* 2002). Even unadapted parents with poor phenotypes can contribute favorable alleles to their progenies when these adapted genotypes are placed in favorable backgrounds. Therefore screening based on phenotypes may miss much of the favorable variation, and thus new allelic variation may be identified by means of markers and can be complimented with phenotypes (Tanksley & McCouch, 1997).

Various DNA based fingerprinting techniques are available for genetic variability studies (Liu & Cordes, 2004; Botha *et al.,* 2004; Kosman & Leonard, 2005). These provide powerful tools in the identification of genetically similar/dissimilar germplasm. In maize, RFLP have been used quite extensively for this purpose (Melchinger, 1993). However, RFLP assays are labour intensive and time consuming and therefore are increasingly substituted by other techniques based on the polymerase chain reaction such as AFLP and simple sequence repeats (SSR). AFLP are genomic fragments detected after selective amplification (Vos *et al.,* 1995). The major advantages of AFLP markers compared with markers like RFLP and SSR are the generation of multiple marker bands in a single assay without prior knowledge of sequence and are highly reproducible (Myburg *et al.,* 2001; Botha *et al.,* 2004). The usefulness of AFLP markers for genetic diversity studies has been demonstrated in many crops (Mackill *et al.,* 1996; Powell *et al.,* 1996; Pejic *et al.,* 1998).

Many maize breeding programs in Africa, for example, in Tanzania, has not yet been studied at a molecular level their breeding germplasm in order to know the extent of genetic diversity which are present in their working gene pools. Such information are important for biotic/abiotic resistance breeding and for the production of varieties/hybrids which are higher yielders and resistant to different stresses. Furthermore, such information could also help to show the composition of genetic variation in these materials as a strategy to identify sources of biotic and abiotic stress resistance. Finally, studies of this nature could help to suggest appropriate measures that should be taken to sustain the existing genetic variation and against depletion of the present gene pools. In this study, the genetic distances of inbreds using AFLPs were determined and thereafter intend to use this information to assess the level of genetic diversity of the Tanzanian gene pools. Also, to test the usefulness of AFLP for determining best line combiners for increased hybrid heterosis. Lastly to predict the potential of these inbreds to produce high yielding GLS resistant hybrids for commercial use.

Materials and methods

Plant material

Twenty-one inbred lines were selected from the maize breeding program in the Southern highlands (1500 and 2500 metres above sea level) of Tanzania which is located in the Mbeya region. This region produces more than 50% of the country's maize annual output (Lyimo per. comm.). These inbreds were analysed using AFLP analysis (Table 1). The selection of the lines was based on their previous performance in a screening test for GLS resistance. Three seeds of each inbred line were sown in a plastic pot containing approximately 1.5 kg mixture of four parts sterilized clay soil and one part sand soil. Approximately 120 grams of a compound fertilizer of N: P: K (2:3:2) was applied to each pot. This pot trial was conducted at the University of Pretoria Experimental farm during the 2000/2001-rain season. At the three to four leaf stage (when plants were about two weeks old), the youngest fresh leaves of each line were harvested and immediately put on ice for DNA extraction.

DNA extraction

Genomic DNA was extracted from leaves of each of the lines as described by Doyle & Doyle (1987). Total genomic DNA was extracted from approximately 100 mg of leaf tissue using 5% (w/v) Cetyltrimethyl ammonium bromide (CTAB) [0.1M Tris-HCL (pH 8.0), 1.4 MNaCl, 20 mM EDTA (pH 8.0), 1% (w/v) soluble Polyvinylpyrrolidone (PVP) and 0.2 % (v/v) 2-Mercaptoethanol). Genomic DNA of each inbred was precipitated by using either ice-cold isopropanol or 95 % ethyl alcohol. The DNA pellets were dissolved in either 100 µl double distilled water or in low TE buffer [10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA] and then stored at –20 ^{0}C. Quantification of the genomic DNA was done using a spectrophotometer.

Table 1. Origin, pedigree and heterotic groups of maize lines used in this study.

N0	Name of a line	Kernel type	GLS Rating	Source of germplasm	Pedigree	Established heterotic group
1	K53015213	Flint	3.25	K205xK230	K530 $S_5$152-1-3	A
2	K5301482	Flint	3.00	K205xK230	K530 $S_5$1482 (97)-1	A
3	K53014821	Flint	2.25	K205xK230	K530 $S_5$14821(98)-1	A
4	P629521	Flint	2.75	Population 62	P62 s_7-95-2-1	A
5	P621111	Flint	2.50	Population 62	P62 S_7 11-1 –1	A/B
6	P627733	Flint	2.00	Population 62	P62 $s_6$77-3Pap3	A
7	P62L50	Flint	2.25	Population 62	P62 $s_6$50	A
8	P62145	Flint	1.50	Population 62	P62$s_6$145-95	A/B
9	CML37	Dent	2.25	Population 32	Pop32c4Hc128-1-1-B-H5	A
10	P621621	Flint	1.75	Population 62	P62$S_6$6-2-1 Pap2	A
11	P628495	Flint	2.50	Population 62	P62 $s_6$84-95Blk	A/B
12	P62103	Flint	8.00	Population 62	P62 103s_6-93	A
13	CML11	Dent	3.00	Population 21	Pop21c5hc219-3-2-2-3#7-1B-4-10b	A
14	E50932815	Dent	2.25	E250xE393	E5093 s_6Pap28-1-5	B
15	P621321	Flint	1.75	Population 62	P62 $s_7$13-2-1	A
16	K375891	Dent	2.50	K337xK358	K3758 $S_7$9-1-1-1	A
17	P62 101	Flint	3.50	Population 62	P62 101-95	A
18	P62148	Flint	2.50	Population 62	P62 $s_6$148-95	B
19	E5093642	Dent	3.50	E250xE393	E5093 $s_7$64	B
20	K37581011	Dent	3.25	K337xK358	K3758 $S_7$10-1-1	A
21	K3758L36	Dent	2.00	K337xK358 L393	K3758s_6L36	B

AFLP analysis

AFLP analysis was performed according to the protocol of Vos *et al*. (1995). Genomic DNA of the maize inbreds (approximately 100mg) was digested with restriction enzymes *EcoR*1 and *Mse*1. In a second step the following adaptor sequences were ligated to the restricted DNA fragments.

*EcoR*1: 5'-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5'

*Mse*1: 5'-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5'

The primers used for pre-amplification and amplification were similar to those described by Vos *et al.* (1995) with *EcoR*1/*Mse*1 extensions ACA/CTG, ACA/CAG, ACA/CCG, ACA/CCC and ACA/CGC (Table 2). The five best primer combinations out of 10 screened were chosen for the final selective amplification. *EcoR*1 primers were 5' labelled with infrared dye (1 µM IRDye700 or IRDye 800, LI-COR, Lincoln, NE, USA). The PCR cycle profile for selective amplification was as follows: one cycle of 94 ^{0}C for 10s, followed by 13 cycles of 65 ^{0}C for 30s, with 0.7 ^{0}C decrease/cycle. Then there was 23 cycles of 94 ^{0}C for 10s, 56 ^{0}C for 30s and 72 ^{0}C for 1 min, with 1s decrease /cycle. Finally, one cycle of primer elongation at 72 ^{0}C for 1 min.

Table 2. The five *EcoR*1/*Mse*1 primer pair combinations used in this study. *EcoR*1 was employed in combinations with the five *Mse*1 primers.

Name of primer	Primer sequence						
ACA-700	5' GAC	TGC	GTA	CCA	ATT	CAC	A-3'
*Mse*1-1	5'GAT	GAG	TCC	TGA	GTA	ACC	C-3'
*Mse*1-2	5'GAT	GAG	TCC	TGA	GTA	ACT	C-3'
*Mse*1-3	5'GAT	GAG	TCC	TGA	GTA	ACA	C-3'
*Mse*1-4	5'GAT	GAG	TCC	TGA	GTA	ACC	C-3'
*Mse*1-5	5'GAT	GAG	TCC	TGA	GTA	ACG	C-3'

Electrophoresis and image analysis

AFLP fragments were resolved in 8% LongRanger^{TM} polyacrylamide gels using the LI-COR IR2 automated DNA analyser (LI-COR, Lincoln, NE, USA). The gel images were scored in a binary matrix that recorded the presence of bands as a plus (+) and absence of bands as minus (-). Semi-automated scoring was performed with the SAGA^{MX}software (Version 3.23, LI-COR). Scores were also manually edited to make corrections to the automated score where necessary.

Data analysis

Polymorphic bands were scored as plus (+) and minus (-) and converted to 1 or 0 when compiled into a data matrix. The data matrix was used to perform cluster analysis on the basis of the average linkage method, known as the Unweighted Pair Group Method (UPGMA) using PAUP software (Swofford, 1993), while the "goodness of fit" of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between dissimilarity and the cophenetic matrix derived from the dendrogram (Sneath & Sokal, 1973). Polymorphism information content (PIC) provides information on the informativeness of a marker or an estimate of the discriminatory power of the locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values in this study were calculated by algorithm: PIC or (the diversity index of Nei, 1973) =1- $\sum_{i=1}^{n} fi^2$ where f is the frequency of ith allele averaged across loci. The marker index (MI) was calculated for the AFLP markers by applying the formulae given by Powell *et al.* (1996) and Smith *et al.* (1997). MI = % polymorphism x PIC and % polymorphism = number of polymorphic bands/ total number of bands in that assay unit.

Calculation of coefficient of coancentry (f)

The estimates of coefficient of coancentry (f) were taken from Messmer *et al.* (1992), wherein f was calculated as described by Falconer & Mackay (1996). For all pair of lines without known parentage, f was set to zero. Two lines were designated as unrelated if their coancentry was smaller than 0.1. Accordingly, for two inbreds related by pedigree with coancentry f and unknown marker genotypes of their ancestors an estimates of their GDs can be obtained as: $\hat{GD} = \overline{GD}$ (1- f), and if the GD of two individuals is known their coancentry can be estimated by: $\hat{f}$ =1-GD/$\overline{GD}$ where $\overline{GD}$ refers to the mean genetic distance of unrelated homozygous lines from the respective germplasm group with regard to the investigated set of marker loci and GD is the genetic distance between the two individuals.

Estimation of genetic distances

Estimates of GD among the 21 line combinations within each set were computed for AFLP marker system using the formula given by Nei & Li (1979). $GD_{ij}= (N_i+N_j-2N_{ij})/(N_i+N_j)$. Here GD_{ij} is the genetic distance between two inbred lines i and j. N_{ij} is the number of common bands in line i and j, and N_i and N_j are the total number of bands in line i and j respectively, with regard to all primer pair combinations of AFLP. Thus, GD reflects the proportion of bands in common between the two inbred lines and it may range from 0 (identical profile for two inbreds) to 1 (no common bands).

The Dendrogram

UPGMA was used to produce the dendrogram on the basis of AFLP markers and 21 inbreds (Figure 1), rather than Ward since the main aim of this research was to study the over all pattern of genetic diversity and not to maximize distance between the morphological traits as in case of populations. Warburton *et al.* (2002) used UPGMA to analyze CIMMYT lines for the same reason.

Results

Degree of polymorphism

The level of polymorphism was measured by the number of polymorphic bands, percent polymorphism, polymorphic index content, and marker index as shown in Table 2. The average marker index and the average PIC values for all five primer combinations were 21.4 and 0.26, respectively. Results showed that the largest marker index (24.9) was revealed by the primer pair combination *EcoR*1-ACA+*Mse*1-CCC while the assay unit *EcoR*1-ACA+*Mse*1-CGC showed the lowest index (16.5). The marker indices for the remaining assay units were in between. The total number of fragments (monomorphic and polymorphic bands) per primer pair combination varied from a minimum of 38 (primer pair combination *EcoR*1-ACA+*Mse*1-CGC) to a maximum of 67 fragments (primer pair combination *EcoR*1-ACA+*Mse*1-CCC). The total number of all AFLP markers in this study was 250 fragments, of which 208 fragments were polymorphic and the fragment sizes ranged from 50 base pairs to 456 base pairs (primer combinations not shown). Furthermore, results showed that the highest PIC (0.30) value was showed by the primer pair combination *EcoR*1-ACA+*Mse*1-CAG and primer pair combination *EcoR*1-ACA+*Mse*1-CGC revealed the lowest PIC (0.22) value. The PIC values for the remaining primer pair combinations were in between.

Table 3. Degree of polymorphism and information content for five primer combinations applied to 21 Tanzanian lines.

*EcoR*1	*Mse*1	Total fragments	Polymorphic bands	%Polymorphism	PIC	MI
1.ACA	CCC	67	58	86	0.29	24.9
2.ACA	CTG	40	34	84	0.26	21.8
3.ACA	CAG	50	40	80	0.30	23.2
4.ACA	CCG	55	47	85	0.24	20.4
5.ACA	CGC	38	29	75	0.22	16.5
Total		250	208	-	-	-
Average		50	41.6	82	0.26	21.4

MI=Marker index = % Polymorphism x PIC.

% Polymorphism= (Polymorphic bands x 100)/ Total fragments.

Genetic distance between inbred combinations of related and unrelated lines

Comparison of GD estimates between inbreds from the same and different heterotic groups were restricted to pairs of unrelated ($f = 0$) lines to minimize confounding effects due to relatedness (Table 4)**.** Genetic distance estimates for the 210 line combinations of 21 inbreds ranged from a minimum of 0.14 (intrapopulation) to a maximum of 0.45 (interpopulation) with a standard deviation for individual estimates varying from 0.02 to 0.06, respectively. Genetic distance estimates for unrelated line combinations within population 62 ranged from 0.14 to 0.33; of type K530 x K530 was from 0.15 to 0.18 and K3758 x K3758 was between 0.17 and 0.20 with a mean of 0.23, 0.16 and 0.19, respectively. Estimates of interpopulation line combinations varied from a minimum of 0.16 to a maximum of 0.45. Population 62 varied in their mean GD to K530 lines from 0.16 to 0.23 and to K3758 from 0.19 to 0.23 as well. Generally, (Table 4) the mean genetic distance of interpopulation (crosses between heterotic groups) (0.32) was much higher than the mean GD of crosses in intrapopulation groups (0.19) (crosses within heterotic groups).

Table 4. Summary of mean, maximum and genetic diversity of individual line combinations calculated from AFLP data using five primer combinations for various groups of related and unrelated pairs of 21 inbred lines.

Type of population	Crosses	#	Mean	Minimum	Maximum	SD	SE
A. Intrapopulations	1. Population 62 x Population 62	44	0.23	0.14	0.33	0.05	0.008
	2. K530 x K530	3	0.16	0.15	0.18	0.02	0.01
	3. K3758 x K3758	3	0.19	0.17	0.20	0.02	0.01
Mean (Intrapopulation)			0.19	0.15	0.23		
B. Interpopulations	1. Population 62 x K530	30	0.23	0.17	0.30	0.04	0.007
	2. K530 x K3758	9	0.24	0.16	0.27	0.04	0.01
	3. Population 62 x CML lines	20	0.31	0.25	0.36	0.03	0.006
	4. K530 x CML lines	6	0.25	0.16	0.32	0.06	0.02
	5. Population 62 x K3758	33	0.28	0.16	0.40	0.06	0.01
	6. Population 62 x Ecuador	22	0.32	0.21	0.38	0.05	0.01
	7. K530 x Ecuador	6	0.35	0.31	0.38	0.02	0.03
	8. K3758 crosses	12	0.34	0.25	0.42	0.06	0.01
	9. CML crosses	6	0.39	0.31	0.43	0.06	0.02
	10. Ecuador crosses	6	0.45	0.45	0.45	-	-
Mean (Interpopulation)			0.32	0.24	0.37		
Total		200					

= refers to the total number of individual crosses in that particular cross

Correlation coefficients between GD and coancentry coefficient (f)

The coancentry coefficient (f) and GD estimates represent fundamentally different concepts for measuring genetic diversity of related individuals (Table 4). Results showed that the coefficient of coancentry (f) for the flint type inbreds was 0.14, while the dent type lines exhibited a f value of 0.20. Furthermore, correlation coefficients between GD and the f values revealed that the flint type inbreds had a correlation value of –0.96. While the dent type inbreds produced a correlation coefficient between GD and coefficient of coancentry of (-0.94) respectively.

Table 5. Coefficient of coancentry and correlation coefficients (r) between genetic distances and coancentry coefficients (f) of maize lines.

Maize germplasm	Number	f†	r(GD, f)	Marker used
Pop 62 (Flint)	5PC	0.14	-0.96	AFLP
K3758 (Dent)	5PC	0.20	-0.94	AFLP

† = Mean of (f) values for all pairs considered.

PC= Number of primer pair combinations used

UPGMA dendrogram

The dendrogram or the genetic similarity/dissimilarity tree produced in this study is presented in Figure 1. In this dendrogram, there are three main distinct groups of inbred similarities. There is group 1. The lines in this group are: K53015213, K5301482 and K53014821**.** Group II, which is the largest group was comprised of lines developed from population 62. These lines were: P629521, P627733, P62L50, P628495, P62103, P62101, P62148, P621111, P621621, P621321 and lastly P62145. There were four subgroups of lines within group II. These subgroups were: Subgroup 1 (lines P629521, P627733, P62L50 and P628495). Subgroup 2. (lines P62103, P62101 and P62148). Subgroup 3 (lines P621111, P621621 and P621321). Then lastly, P62145 was an outlier in this main group. The third main group was comprised of lines which originated from a recombinant line K337 x K358. These lines were: K3758911, K37586911 and K37581011. Finally, the dendrogram revealed that the CIMMYT lines (CML37 and CML11) and the Ecuador lines (E50932815 and E5093642) were also outliers.

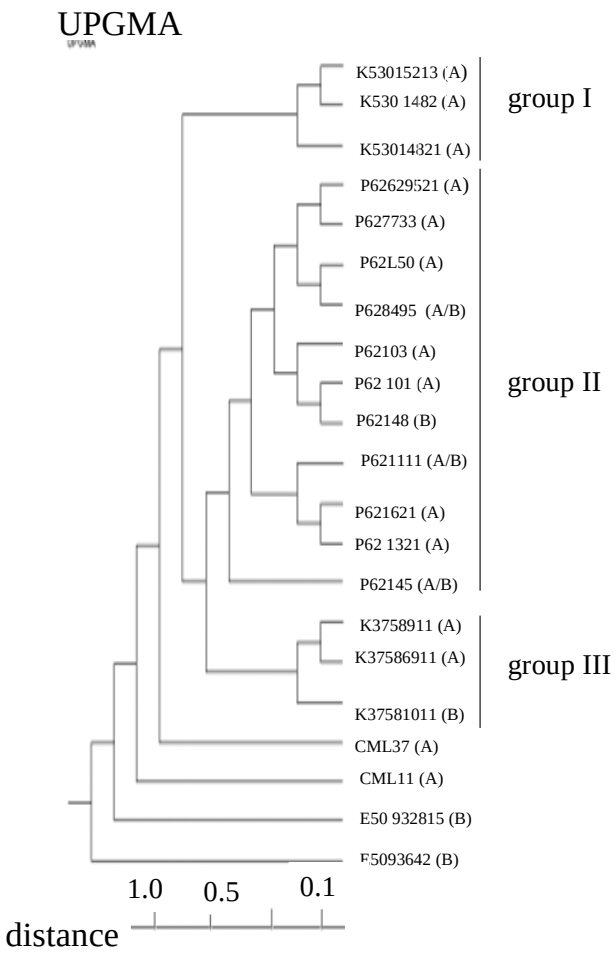

Figure1.The UPGMA dendrogram showing the grouping of twenty-one inbreds used in this study.

Discussion

In general, each of the five AFLP primer combinations used in this study discriminated effectively between most of the unrelated lines. The average marker index in this material was 21.4 (Table 3) which was slightly higher than that reported by Lübberstedt *et al.* (2000) in European maize. Their marker index was 16.4. The larger marker index in this study was attributable to the high average percent of polymorphic bands per lane, while the PIC values were about equal or slightly lower than those of Lübberstedt *et al*. (2000).

Similar results were reported for US maize germplasm (Pejic *et al.*, 1998), soybean (Powell *et al.*, 1996) and wheat (Bohn *et al.*, 1999). The higher marker index and PIC value of the AFLP markers in comparison with other marker systems like SSR, RFLPs, RAPDs, together with high reproducibility proves that AFLP markers are a valuable tool for identification of maize inbreds, plant variety protection and registration as well as patenting germplasm (Melchinger *et al.*, 1998a, b; Lübberstedt *et al.*, 2000). AFLP have added advantages in terms of reliability, reproducibility, discrimination, standardization and cost effectiveness. Furthermore, the results showed that primer pair *EcoR*1-ACA+*Mse*1-CAG was the most discriminative or informative because it revealed the highest PIC value (0.30) and primer pair *EcoR*1-ACA+*Mse*1-CGC was the least discriminative because it had the lowest PIC value (0.22).

One of the aims of this study was to test and evaluate inbreds as potential parents for developing commercial hybrids. It was demonstrated in the study that the GDs of genotypes from different germplasm groups (intergroup crosses) have on average significantly greater GDs than combinations of genotypes from the same germplasm group (intragroup crosses) (Table 4). This observation is consistent with the genetic theory, and those of Melchinger *et al.* (1998a, b) and Pejic *et al.* (1998) which states that intergroup crosses exhibit higher genetic distances between lines and lower genetic distances between lines in the intrapopulation crosses.

Breeders have used the coancentry [devised by Malecot's (1948)] as an indirect way of measuring genetic similarities between related individuals. It was observed that r (GD, f) values (Table 5) in both flint and dent kernel lines were much higher than in maize (Messmer *et al.*, 1993; Smith *et al.*, 1997) and other reported crops (Graner *et al.*, 1994; Schut *et al.*, 1997). The negative sign of the r (GD, f) means that in this case as one trait increases, the second tends to decrease. The main reasons for the higher r (GD, f) in this study could be due to fundamental differences between the two marker systems used. The AFLP marker system used in this study scores bands on a biallellic basis, while Messmer *et al*. (1993) and Smith *et al.* (1997) used RFLP, which usually scores multiple bands (alleles) per locus (Lübberstedt *et al.*, 2000). Secondly, the AFLPs are capable of

assaying more genetic loci than RFLP or SSR (Myburg *et al.*, 2001; Botha *et al.*, 2004; Liu & Cordes, 2004). Thirdly, the higher r (GD, f) could be due to the fact that AFLPs are able to amplify potentially more conserved organellar DNA sequences than the RFLP (Vuylsteke *et al*., 1999). Melchinger (1993) reported that high r (GD, f) estimates in maize suggest that if the pedigree data are correct, these correlations (GD, f) do provide reliable descriptors of genetic similarity in maize.

The dendrogram (Figure 1) revealed that the three recombinant inbreds, developed from K530 germplasm, clustered together and showed some level of genetic similarity with group II, which was comprised of inbreds originating from population 62. This implies that there are some genetic similarities between the recombinant inbreds derived from the K530 germplasm and those developed from population 62. This could be due to gene introgressions between the two sources of germplasm or between the inbreds *per se* or both. The intermingling of genes between the two sources could be via deliberate breeding or through contamination or both. These observations are vital to plant breeders in the sense that they should know the background history and the genetic purity of sources of germplasm they are working with. This, in turn, will help the breeders to develop inbreds without linkage drag that happens due to use of contaminated germplasm.

In general, the AFLP method could clearly distinguish all the inbreds and clustered them into appropriate groups according to the established pedigrees. However, there were a few discrepancies, especially in the placement of individual inbreds according to the source of germplasm from which they have been extracted. For example the grouping of all recombinant inbreds that were developed from K337 x K358 germplasm clustered with the group containing inbreds extracted from population 62. It was also observed that there were a lot of subgroups of inbreds derived from population 62, implying that this population is genetically diverse. Furthermore, line P62145, although clustered in-group II, did not cluster with any of the subgroups. Similar discrepancies of clustering of melon inbreds by using SSR within one germplasm source have been reported between population of origin and heterotic grouping, a result consistent with high level of genetic

diversity within source populations of inbreds (Monforte *et al*., 2003). The CIMMYT lines, which have different sources of germplasm, were grouped as expected. The Ecuador lines, however, according to their pedigree were supposed to group together but fell into different groups. This could be due to contamination or small mutations occurring in one of these lines.

Conclusions

AFLP markers for genetic diversity studies in maize have been employed to investigate the relationship between GD and hybrid performance/heterosis for yield and MPH (Melchinger *et al.*, 1998a, b; Lübberstedt *et al.*, 2000). In this study, however, it was observed that the AFLP markers have another added advantage of producing the best results for measuring correlations between coancentry coefficient and genetic distance of maize inbreds. It had the highest f values as compared to similar studies reported by Smith *et al.* (1997) and Messmer *et al.* (1993) etc. Furthermore, this study revealed that each line was uniquely identified when the five AFLP primer combinations were used and the grouping of the lines was almost consistent with their established pedigree (Figure 1). This research also showed that inbreds derived from intrapopulations as well as recombinants can have lower genetic distances than inbreds derived from open pollinated varieties (Table 4) and might suffer from the deleterious effect of inbreeding depression and degeneration. Finally, it is important to note that inbreds which are similar can only be crossed among themselves if (i) they can show an increased level of heterosis of the target trait; (ii) these inbreds could complement one another for those genes which are missing in each other and these genes are able to improve the agronomical traits of the resulting hybrids; and (iii) are needed to produce large quantities of seed which single crosses are not able to produce.

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CHAPTER 4

ASSOCIATION BETWEEN AFLP- BASED GENETIC DISTANCE AND HYBRID PERFORMANCE IN THE TANZANIAN MAIZE INBREDS

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Abstract

The identification of best inbred combinations for the development of commercial hybrid maize varieties is still the main challenge to maize breeders. Several strategies including crossing parents from different heterotic groups, pedigrees, and/or testers have been attempted. The main pitfall of these methods is that they are mainly based on phenotypic data, which are confounded by environmental variations. Thus the main aim of this study was, therefore, to study the associations between amplified fragment length polymorphisms (AFLPs) based genetic distance (GD) and F_1 phenotypic data. Furthermore, the efficacy of grouping lines into genetically similar clusters was investigated. This research used 21 inbreds, which were fingerprinted using AFLP analysis and their 210 F_1 progenies were planted in the field. Results of the analysis were applied to predict the best line combinations for commercial maize hybrid production. Joint data analyses revealed a tighter association between GD and the F_1 traits or mid parent heterosis (MPH) in the intergroup than in the intragroup crosses. Intergoup crosses should be field tested before their release. Intragroup and intergroup crosses showing low GD-MPH might be discarded to avoid field-testing costs. Better F_1 hybrid performance predictions can be achieved by integrating molecular and F_1 phenotypic data.

Keywords: Genetic distance, intragroup, intergroup, maize, phenotypic traits, heterosis.

Introduction

Genetic diversity among breeding materials is very important in plant breeding, as it largely determines the future prospects of the breeding program (Hallauer *et al.*, 1988). Before 1970, genetic diversity in crop plants was determined based on pedigree data (Lübberstedt *et al.*, 2000) and morphological traits (Yee *et al.*, 1999). To date, some of the genetic diversity study methods applied in crops include Malecot's (1948) coefficient of coancentry (f). This method however, is not commonly used because of several assumptions that are not always fulfilled (Messmmer *et al.*, 1993). Distance from geographic origin (Hallauer *et al.,* 1988; Moll *et al.,* 1965) has also been used to compare genetic distances between genotypes. The problem with the distance from geographic origin approach is that more germplasm from one geographic area are mixed or contaminated. Lastly, tester lines A and B is the most commonly and widely used method to assign lines into heterotic groups. But some of the pitfalls of the testers are that (a) some lines show heterosis when crossed to both testers, (b) many lines fall into more than one heterotic group, (c) the two testers are not sufficient to represent the genetic diversity present in the maize heterotic groups, and (d) including more testers can be expensive and time consuming (Warburton *et al.*, 2002).

In crop breeding programs, information on genetic relationships within species is used to organize germplasm collections, identify heterotic groups and to facilitate selections of breeding materials (Lee, 1995; Karp *et al.*, 1996). Several studies have tried to study and relate hybrid performance in maize using phenotypic markers and quantitative traits (Goodman, 1972). This was followed by genetic markers in the form of gene products such as isozyme diversity (Cox *et al.*, 1988; Melchinger *et al.*, 1990) and storage proteins (Cox *et al.,* 1988). However, the use of isozymes in these studies had limitations. The major limitations of the isozyme studies could be due to the low number of loci examined and low number of polymophisms detected (Melchinger *et al.*, 1990; Senior *et al.*, 1998). Direct DNA markers (Genetic markers) such as restriction fragment length polymorphisms (RFLPs) in these studies were suggested and applied to overcome the constraints associated with isozymes (Burr *et al.*, 1983).

The choice of inbred lines as sources of favourable genes for hybrid production and performance is of major concern to breeders. Different germplasm sources are screened for the presence of favourable genes. Pedigree, morphology and agronomic characters have been used to establish the description of a genotype (Jarman and Pickett, 1992; Martin *et al.*, 1998). However, phenotypic data may poorly reflect actual levels of genetic diversity among genotypes (Yee *et al.*, 1999). This is because such methods are subjective and are often influenced by environmental conditions (Russell *et al.*, 1997; Rafalski *et al.*, 1996). There is also greater level of heterosis expressed when genetically dissimilar parents are crossed (Martin *et al.*, 1998; Cheres *et al.*, 2000).

The DNA marker systems are useful tools for assessing genetic diversity among germplasm groups. The development of molecular markers started with the RFLP marker system in the 1980's (Bostein *et al.*, 1980). RFLP markers were regarded as the first shot in the genome revolution (Dodgson *et al.*, 1997). This technique, however, is not commonly used as it is too complex, require large amount of DNA and relatively more expensive than polymerase reaction (PCR) based (Tragoonrung *et al.*, 1992) marker systems. The PCR based marker systems like AFLP and simple sequence repeats have provided new tools for heterosis prediction and to investigate correlations between parental GDs and the performance of F_1 hybrids or MPH. AFLPs have been tested and confirmed as being high quality markers, highly reproductive and highly informative (Vos *et al.*, 1995; Liu and Cordes, 2004).

Information about the relationship among breeding materials and the genetic diversity in the available germplasm is important for the choice of parents in the plant breeding programs which apply particularly to hybrid breeding, where recognition and exploitation of heterotic patterns between different types of germplasm are important for success. How divergent are the lines from the same and different germplasm pools is one of the most fundamental questions for breeders.

The associations between genetic distances of maize inbred lines and the performance of F_1 phenotypic traits of agronomic importance including GLS scores, where a score of one refers to maize genotypes which are highly resistant to the GLS disease,

whereas nine corresponds to maize genotypes that highly succumbed to the GLS pathogen (Donahue *et al.*, 1991). These factors in maize are important driving forces for the production of commercial hybrid varieties. The relationships of these factors as determinants in maize breeding have not been studied or described in detail, neither have their applications for maize improvement in breeding programs. Also, this study assessed the efficacy of AFLP markers for grouping lines into genetically similar clusters and tested the genetic diversity of these lines in the intragroup and intergroup crosses. Furthermore, pairwise genetic distances of the inbred lines were compared, genetic distances of the lines with performance of phenotypic traits of their crosses were correlated, and finally, the intra and intergroup crosses were characterized in order to generate important information which could help maize breeders identify best line combinations for the production of commercial maize hybrid varieties.

Materials and methods

Plant material and Field design

Twenty-one inbred lines from the High Altitude Maize Improvement Program of Tanzania, (HAMIPT), which is located in the Mbeya region, in the Southern Highlands zone of the country (Table 1) were used in this study. The twenty-one lines were crossed in a complete diallel mating design. The field work was conducted for three consecutive years (2003-2005) across three locations (nine environments). There were 225 hybrids in total (210 single crosses, two controls and 13 filler materials) which were planted using a 15 x 15 triple lattice, and the inbred lines were planted in a randomized complete block design with three replications at each site. Planting was done in the mid December every year in all the sites. The plot size was a single row plot, 5.1 m long with inter row spacing of 0.75 m and intra row spacing of 0.30 m. Two kernels were planted in each hill, thereafter plants were thinned to one plant per hill, and this resulted in a plant population size of 44,400 plants/ha.

Morphological traits

The performance of F_1 phenotypic traits recorded included: yield, days to 50 % silking, height to ear, ear length, kernels/ear, rows/ear and GLS score. The grain

yield/plot was measured in terms of kilograms/ha at grain moisture content of 12.5 percent. Days to 50 % silking was obtained by counting number of days from the day of planting to the day when 50 % of the plants in each plot have silks. Height to ear was measured as the average height in centimeters from the ground level to the first ear in each plot. Ear length was measured as the average length in centimeters of all ears in each plot. The number of rows/ear was obtained by counting the number of rows/ear for all ears in a plot. The average number of rows/ear was taken as the overall number of rows/ear of that plot. The number of kernels/row was obtained by counting the number of kernels/row for all ears of a plot. The average number of kernels/row was taken as the overall number of kernels of that plot. Gray leaf spot scoring was recorded on a scare of 1-9 according to Donahue *et al.* (1991) with an increment of 0.25. A genotype with a score of one was highly resistant, while a score of nine was highly susceptible.

Furthermore, this study used 45 intergroup crosses (five crosses from each of the following line combinations: CML11 x Population 62 lines, CML11 x K530 lines, CML11 x K3758 lines, CML37 x Population 62 lines, CML37 x K3758 lines, Ecuador 509642 line x Population 62 lines, Ecuador 509642 line x K530 lines and Ecuador 509642 line x K3758 lines) and 45 intragroup crosses (15 intergroup crosses from each of the following line x line crosses; Population 62 x Population 62 lines, K530 x K530 lines and K3758 x K3758 lines) to study the associations between the estimated GD of lines (Table1) and the performance of F_1 phenotypic traits of line x line pairwise crosses (Tables 5 and 6).

Genotyping

The twenty-one inbred lines from the HAMIPT were analysed genetically using AFLP fingerprinting. Three seeds of each inbred line were sown in a plastic pot containing approximately 1.5 kg mixture of four parts sterilized clay soil: and 1 part sandy soil (4:1). Approximately 120 grams of a compound fertilizer of N: P: K (2: 3: 2) was applied in each plastic pot. This pot trial was conducted at the University of Pretoria Experimental farm during the 2000/2001-rain season. At the three to four leaf stage

(when plants were about two weeks old), the youngest fresh leaves of each line were harvested and immediately put on ice for DNA extraction.

DNA Extraction

Genomic DNA was extracted from leaves of each of the lines as described by Doyle and Doyle (1987). Total genomic DNA was extracted from approximately 100 mg of leaf tissue using 5 % (w/v) Cetyltrimethyl ammonium bromide (CTAB) [0.1 M Tris-HCL (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 1 % (w/v) soluble Polyvinylpyrrolidone (PVP) and 0.2 % (v/v) 2-Mercaptoethanol). Genomic DNA of each inbred was precipitated by using either ice-cold isopropanol or 95 % (v/v) ethyl alcohol. The genomic DNA pellets were dissolved in either 100 µl double distilled water or in low TE buffer [10 mM Tris-HCL (pH 8.0), 1 mM EDTA] and then stored at –20 ^{O}C. Genomic DNA was quantified spectrophotometically.

DNA analysis

The genetic analyses used the AFLP protocol (Vos *et al.*, 1995) with minor modifications. Genomic DNA of each maize inbred line (approximately 250ng of DNA) was digested at 37 ^{O}C for 2h using 1.25 U *Eco*RI and *Mse*I restriction endonucleases. Following adapter ligation, preselective and selective amplification reactions were performed. The preselective amplifications were carried out in a 35 µl volume containing 10 µl diluted ligation product, AFLP pre-amplification primer mix (10 µM *Eco*RI and 10 µM *Mse*I primers, 2.5 mM dNTPs each), 10x PCR buffer, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Promega Molecular Chemicals) for 30 cycles with the following cycle profile: adapter extension for 10 s at 72 ^{O}C followed by denaturation for 10 s at 94 ^{O}C, annealing for 30 s at 56 ^{O}C, and extension for 1 min at 72 ^{O}C with a 1 s per cycle increase in extension time. The final selective stage had the *EcoRI*/*Mse*I extensions of: ACA/CCC, ACA/CTG, ACA/CAG, ACA/CCG and ACA/CGC. AFLP fragments were resolved in 8 % long Ranger^{TM} polyacryalamide gels, using the LI-COR IR automated DNA analyser (LI-COR, Lincoln, NE, USA). The gel images were scored in a binary matrix that recorded the presence of bands as a plus (+) and absence of bands as minus (-). Semi automated scoring was performed

with SAGA^{MX} software (version 3.23, LI-COR, NE, USA). Scores were also manually edited to make corrections to the automated score where appropriate.

Table 1. Origin, pedigree and heterotic patterns of maize lines used in this study.

No	Line name	Kernel type	GLS Rating	Germplasm source	Pedigree	Established heterotic gr
1	P62145	Flint	1..5	Population 62	$P62s_7145$-95	A/B
2	K53015213	Flint	3..25	K205 x K230	$K530s_71521$-3	A
3	K375891	Dent	2..50	K337 x K358	$K3758s_7$9-1-1	A
4	K37581011	Dent	3..25	K337 x K358	$K3758_{S6}$810-1-1	A
5	K5301482	Flint	3..00	K205 x K230	$K530s_5$14821(98)-1	A
6	P627733	Flint	2..00	Population 62	$P62s_6$77-3Pap3	A
7	K3758L36	Dent	2..25	K337 x K358	$K3758s_7$L-3-6	B
8	K53014821	Flint	2..25	K205 x K230	$K530s_5$1482-1	A
9	CML37	Dent	2..25	Population 32	Pop32c4Hc128-1-1-B-H5	A
10	P629521	Dent	2.75	Population 62	$P62s_7$95-2-1	A
11	CML11	Dent	3..00	Population 21	Popc5hc219-3-2-2-3#7-1B-4-10b	A
12	P621621	Flint	1.75	Population 62	$P62s_7$162-1Pap2	A
13	P621111	Flint	2..50	Population 62	$P62s_7$11-1-1	A/B
14	P62L50	Flint	2..25	Population 62	$P62S_6$ 50	A
15	P628495	Flint	1.75	Population 62	$P62s_6$84-95Blk	A/B
16	P62103	Flint	8..00	Population 62	$P62103s_8$6-95	A
17	P62101	Flint	3..50	Population 62	$P62s_6$101-95	A
18	P62148	Flint	2..50	Population 62	$P62s_7$14-8 -95	B
19	E50932815	Dent	2.50	E250 x E393	E5093s5Pap28-1-5	B
20	E5093642	Dent	3.50	E250 x E393	$E5093s_7$64	B
21	P621321	Flint	1.75	Population 62	P62s713-2-1	A

E=Ecuador

Data analysis

Phenotypic correlation coefficients (r) between GDs and F_1 phenotypic data were computed using the formula by Dale van Vleck *et al.* (1987). Mid parent heterosis (MPH) and high parent heterosis (HPH) were calculated by the formula of Falconer and Mackay (1996). The experimental F_1 phenotypic grand mean of each trait was compared to the line cross means for producing the phenotypic data matrix (+ and -). When the line cross mean was equal or more than the trait grand mean then a + sign (band presence) plus was assigned to it. Whereas a - minus sign (band absence) was used if the line cross was less than the grand mean (Table 2). Then the phenotypic matrix was added to the inbred line polymorphic bands which were also scored as plus (+) for the presence of the band and minus (-) for the absence of the band and then were converted to 1 or 0 when compiled into a data matrix. The data matrix was used to perform cluster analysis (dendrogram) and pairwise genetic distances of the 21 inbred lines using the NCSS 2001 software program (Jerry, 2001) or the pairwise line

genetic distances were computed within each set of the AFLP marker system using the formula given by Nei and Li (1979), which is $GD_{ij} = (N_i + N_j - 2N_{ij})/(N_i + N_j)$; where GD_{ij} is the genetic distance between two inbred lines i and j. N_{ij} is the number of common bands between line i and j, and N_i and N_j are the total number of bands in line i and j, respectively, with regard to all primer pair combinations of AFLP. Thus, GD reflects the proportion of bands in common between the two inbred lines and it may range from 0 (identical profiles for two lines) to 1 (no shared bands). The "goodness of fit" of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficients between dissimilarity and cophenetic matrix derived from ther dendrogram (Sneak and Sokal, 1973). Polymorphism information content (PIC) which provides an estimate of the discriminatory power of the locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles was computed for each marker assay unit. PIC values in this study were calculated using the algorithm: PIC or (the diversity index of Nei, 1973) = $1- \sum_{i=1}^{n} fi^2$ where f is the frequency of i^{th} allele averaged across loci. The PIC also provides information on the informativeness of a marker system. Percentage polymorphism was calculated using the following formula: number of polymorphic bands/total number of bands in that assay unit x 100.

Table 2. The experimental grand means of F_1 phenotypic data of each trait used to produce the F_1 phenotypic data matrix in this study.

Trait	(+) sign	(-)sign
1. GLS	Score ≤ 3.5 GLS rating	Score > 3.5 GLS rating
2. Yield	Score ≥ 4.8 MT	Score < 4.8 MT
3. Days to 50 % silking	Score ≥ 92.7 days	Score < 92.7 days
4. Ear height	Score ≥ 103.5 cm	Score < 103.5 cm
5. Ear length	Score ≥ 18.5 cm	Score < 18.5 cm
6. Rows/ear	Score ≥ 13.2	Score < 13.2
7. Kernels/row	Score ≥ 39.8	Score < 39.8

Results

The degree of polymorphism was measured by the number of polymorphic bands, percentage polymorphism and polymorphic index content (Table 3). The average PIC

value for the five primer combinations collectively was 0.28, respectively. Results showed that the number of fragments (Polymorphic and monomorphic bands) per primer pair combination varied from a minimum of 42 (primer pair combination *Eco*RI-ACA+*Mse*I-CGC) to a maximum of 74 fragments (primer pair combination *Eco*RI-ACA+*Mse*I-CCC). The total number of all the AFLP markers in this study was 278 fragments, of which 238 fragments were polymorphic with the fragment sizes ranged from 50 base pairs to 456 base pairs (Primer pair combination not shown). Furthermore, results showed that the highest PIC value (0.34) was revealed by the primer pair combination *Eco*RI-ACA+*Mse*I-CAG and primer pair combination *Eco*RI-ACA+*Mse*I-CGC exhibited the lowest PIC (0.20) value. The PIC values for the remaining primer pair combinations were as follows: Primer pair combination *Eco*RI-ACA+*Mse*I-CCC was 0.32, primer pair combination *Eco*RI-ACA+*Mse*I-CTG was 0.28 and primer pair combination *Eco*RI-ACA+*Mse*I-CCG was 0.24.

Table 3. Degree of polymorphism and information content for five primer combinations applied to 21 Tanzanian inbred lines.

*Eco*RI	*Mse*I	Total fragments	Polymorphic bands	% Polymorphism	PIC
1. ACA	CCC	74	68	91	0.32
2. ACA	CTG	48	39	81	0.28
3. ACA	CAG	56	44	78	0.34
4. ACA	CCG	58	52	89	0.24
5. ACA	CGC	42	35	83	0.20
Total		278	238	-	-
Average		56	48	82	0.28

% Polymorphism = (Polymorphic bands/ Total fragments) x 100

Pairwise genetic distance estimates for the 21 inbred lines (i.e. 210 F_1 line combinations) ranged from a minimum of 0.30 (intragroup crosses) to a maximum of 0.63 (intergroup crosses) (Table 4). Genetic distance estimates for line combinations within population 62 lines ranged from 0.33 (line 20 x line 16, line 6 x line 12) to 0.51 (line 1 x line 6) with a mean GD of 0.0.42. While the genetic distance estimates for the K3758 line series showed a minimum GD of 0.31 (line 7 x line 10) and a maximum of GD 0.46 (line 3 x line 4). The mean GD for the K3758 line series was 0.35. Furthermore, for the K530 line series, their GD mean was 0.43, with a maximum GD of 0.57 (line 13 x line 14) and a minimum GD of 0.32 (line 18 x line 14). Finally, data in Table 3 exhibited that such as line 9 x line 8; line 11 x line 12; line 13 x line

12; line 14 x line 16; line 17 x line 16; line 5 x line 6; line 1 x line 2; line 21 x line 20 and line 14 x line 15 recorded the highest line x line pairwise GDs. Whereas combinations like line 15 x line 10; line 7 x line 10; line 3 x line 10; line 4 x line 10; line 12 x line 6; line 17 x line 4; line 17 x line 10; line 18 x line 14; line 19 x line 15 and line 20 x line 16 exhibited the lowest line x line pairwise GD in this study.

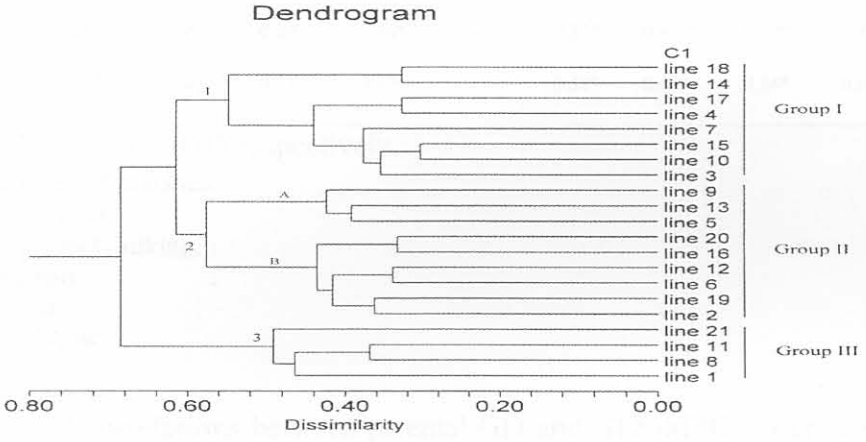
Table 4. Pairwise genetic distance coefficients of lines using five AFLP primer pair combinations on twenty-one lines analyzed by NCSS 2001software program.

	Line 1	Line 2	Line 3	Line 4	Line 5	Line 6	Line 7	Line 8	Line 9	Line 10	Line 11	Line 12	Line 13	Line 14	Line 15	Line 16	Line 17	Line 18	Line 19	Line 20	Line 21
Line1	-	0.61	0.45	0.44	0.47	0.51	0.51	0.49	0.50	0.52	0.38	0.50	0.50	0.46	0.46	0.50	0.44	0.47	0.48	0.53	0.47
Line2		-	0.43	0.46	0.40	0.36	0.42	0.45	0.42	0.42	0.43	0.43	0.42	0.46	0.43	0.37	0.41	0.42	0.36	0.36	0.48
Line3			-	0.46	0.42	0.38	0.40	0.43	0.43	0.33	0.41	0.38	0.39	0.45	0.35	0.39	0.38	0.38	0.39	0.39	0.51
Line4				-	0.51	0.41	0.36	0.36	0.41	0.32	0.36	0.42	0.38	0.41	0.35	0.34	0.32	0.35	0.41	0.35	0.48
Line5					-	0.54	0.50	0.41	0.39	0.40	0.45	0.41	0.39	0.50	0.35	0.44	0.42	0.41	0.39	0.39	0.51
Line6						-	0.47	0.47	0.44	0.36	0.34	0.33	0.48	0.40	0.36	0.37	0.40	0.40	0.39	0.35	0.44
Line7							-	0.52	0.45	0.31	0.38	0.45	0.42	0.39	0.36	0.39	0.40	0.36	0.42	0.41	0.50
Line8								-	0.63	0.43	0.36	0.41	0.41	0.47	0.38	0.43	0.41	0.42	0.45	0.42	0.43
Line9									-	0.52	0.54	0.43	0.43	0.45	0.48	0.43	0.40	0.47	0.40	0.40	0.54
Line10										-	0.47	0.41	0.35	0.41	0.30	0.41	0.32	0.36	0.40	0.34	0.49
Line11											-	0.57	0.43	0.37	0.37	0.41	0.41	0.36	0.42	0.45	0.50
Line12													0.57	0.53	0.39	0.38	0.38	0.42	0.35	0.40	0.41
Line13													-	0.57	0.45	0.39	0.42	0.41	0.48	0.41	0.54
Line14														-	0.54	0.51	0.43	0.32	0.46	0.42	0.57
Line15															-	0.46	0.39	0.38	0.33	0.35	0.43
Line16																-	0.52	0.39	0.42	0.33	0.48
Line17																	-	0.48	0.49	0.35	0.52
Line18																		-	048	0.42	0.50
Line19																			--	0.43	0.46
Line20																				-	0.55
Line21																					-

The dendrogram or genetic similarity or dissimilarity tree produced in this study is presented in Figure 1. This tree revealed three main groups of inbred line-F_1 phenotypic data similarities. Group 1, was comprised of eight line-F_1 phenotypic data in total, of which six lines were K3758 and two lines of K530 series. Group II, however, was the largest one with nine inbred-F_1 phenotypic data. In this group five lines originated from Population 62 germplasm, three lines from K530 inbred series and one CIMMYT line. Furthermore, group II exhibited subgroups, A and B. The subgroup A was comprised of a K530 line, a CIMMYT line and a line from

population 62 germplasm. While subgroup B had five lines from Population 62 and one line belonging to K530 series. Finally, there was group III which had one CIMMYT line one line from K530 series, one line that originated from Population 62 germplasm and the Ecuador line. Inbred lines in group III revealed high genetical diversity as they were from different germplasm sources respectively.

Figure 1. The dendrogram showing the clustering of the 21 inbred lines used in this study.



Results of the associations between the estimated GD of the lines and the performance of F_1 traits of line x line pairwise crosses for the forty five intergroup crosses and the forty five intragoup crosses revealed that yield, GLS score, ear length rows/ear and kernels/row had tighter correlations in the intergroup crosses than in the intragroup crosses (Table 5). But height to ear and days to 50 % silking revealed higher associations in the intragroup crosses than in the intergroup crosses. Furthermore, Table 5 showed tighter correlations between line GD and heterosis for both GLS scores (i.e. GLS-MPH and GLS-HPH), GD and MPH for height to ear, GD and MPH

for days to 50 % silking, GD and MPH for rows/ear and finally GD and MPH for kernels/row in the intergroup than in the intragroup crosses respectively.

Table 5. Correlation coefficients between GD and morphological traits in the intragroup[1] and intergroup[2] crosses and correlation coefficients between GD and their mid parent/high parent heterosis of F_1 morphological traits in both intragroup3 and intergroup crosses[4] in this study.

Type of crosses	#	GD	Yield	GLS	A	B	C	D	E	GLS-HPH	GLS-MPH
Intragroup crosses [1]	45	0.19	0.28*	0.43*	0.59*	0.62*	0.48*	0.27*	0.38*	-	-
Intergroup crosses [2]	45	0.31	0.46*	-0.57*	0.42*	0.51*	0.63*	0.49*	0.56*	-	-
Intragroup Crosses [3]	45	0.19	0.46*	-	0.33*	0.42*	0.69*	0.42*	0.24*	0.21*	0.43*
Intergroup Crosses [4]	45	0.31	0.37*	-	0.51*	0.49*	0.34*	0.68*	0.61*	0.51*	0.48*

* significant at $p = 0.05$ respectively.
# = number of crosses.
A = height to ear.
B = days to 50 % silking.
C = ear length.
D = row/ear
E = kernels/row

The overall correlations between parental GD and GLS-MPH; parental GD and GLS-HPH in maize crop (both inter and intragroup crosses) are exhibited in Table 6. These calculations attempt to elucidate whether MPH or HPH had the tightest association with gray leaf spot resistance in maize. Results indicated that GLS-HPH is more tightly associated with GD than GLS-MPH for GLS resistance. Table 6 also show that correlations between inbred GD and F_1 grain yield, inbred GD and MPH for grain yield. Finally results in this table (Table 6) revealed that r(GD, F_1 grain yield) was 0.39. Both mid parent heterosis (for grain yield) and GLS-MPH exhibited an association of 0.1 with GD of inbred lines, but GLS-HPH showed a tighter correlation coefficient (0.27) with GD of lines.

Table 6. Correlation coefficient r (GD, MPH (yield); GD, GLS-MPH and GD.GLS-HPH.

	F_1 grain yield	MPH (yield)	GLS-MPH	GLS-HPH
r(GD)	0.39*	0.1*	-0.1*	-0.27*

* significant at $p = 0.05$ respectively.

Discussion

In general each of the five primer pair combinations used in this study discriminated effectively most of the unrelated lines. There was a high average percent of polymorphic bands (82 %) per assay unit, and the PIC values were comparable to those of Lübberstedt *et al.* (2000). Similar results were reported for US maize germplasm (Pejic *et al.,* 1998), soybean (Powell *et al.*, 1996) and wheat (Bohn *et al.*, 1999). The higher PIC values of the AFLP markers in comparison with other marker systems like RFLPs, RAPDs Microsatellites, together with higher reproducibility shows that AFLP markers are a valuable tool for identification of maize inbred lines, plant variety protection and registration as well as germplasm patenting (Melchinger *et al.*, 1998; Ajmone-Marsan *et al.*, 1998; Lübberstedt *et al.*, 2000). AFLPs have other advantages in terms of reliability, reproducibility, discrimination, standardization and cost effectiveness. Furthermore, these results showed that primer pair E*coR*I-ACA+*Mse*I-CAG was the most discriminative or informative because it revealed the highest PIC value (0.30) and primer combination E*coR*I-ACA+*Mse*I-CGC was the least discriminative as it had the lowest PIC value (0.22) (Table 3).

The dendrogram in this study revealed that the inclusion of phenotypic data produced different groupings from AFLP genotyping data alone when predicting best line combinations and hybrid heterosis (Kiula *et al.*, 2006). This study showed that the grouping of inbred lines plus phenotypic data gave better predictions of hybrid performances since different dissimilar lines were grouped together (Figure 1). This could be based on the genetic theory that maximum heterosis of F_1 hybrids are products of crossing inbred lines which are genetically dissimilar. Hence genetic analyses of inbred lines with phenotypic data improved the predictions on combining ability for the production of commercial hybrid maize varieties.

This research also evaluated the Tanzanian inbred lines as potential parents for developing commercial hybrids. Thus the study included agronomically important traits such as GLS score, days to 50 % silking, kernels/row, rows/ear, height to ear, ear length and grain yield that have not been observed yet in studies in this context before. It is demonstrated that the GDs of genotypes from different germplasm groups

(intergroup crosses) have on average significantly greater GDs than combinations of genotypes from the same germplasm group (intragroup crosses) (Table 5). This observation is consistent with the current genetic theory and those of Melchinger *et al.* (1998) and Pejic *et al.* (1998) who reported that intergroup crosses exhibit higher genetic distances between lines and lower genetic distances for those lines in the intragroup crosses. Results in this study also showed that there are higher correlations between GD and F_1 or MPH data for intergroup crosses than in the intragroup crosses (Table 5). This observation complied with the expectation of Melchinger (1999) know as "group effects". The group effects occurred because both GD and MPH or F_1 phenotypic data were expected to increase from crosses among related lines to intragroup crosses and further to intergroup crosses. In addition to this, an increase in heterosis in intergroup crosses are due to a more favourable ratio of general combining ability (GCA) to specific combing ability (SCA) variances (Dhillon *et al.*, 1990). However, there are correlations between GD and MPH or F_1 in intragroups that were sometimes tighter than in the intergroup (Table 5). Reports of Charcosset *et al.* (1991) and Lübberstedt *et al.* (2000) supported the obtained results and these observations can be explained by the quantitative-genetic theory (Chacosset and Essioux, 1994) which states that in the intergroup crosses, the covariances between the specific genetic distance and specific combining ability (SCA) is a sum of positive and negative terms, which may cancel each other resulting in low or zero correlation between GD and F_1 or MPH. This is because the maternal and paternal gametic arrays may differ in the linkage phase for many quantitative trait loci-marker pairs (Chacosset and Essioux, 1994). The tighter associations of GD with MPH or F_1 in Table 5 in the intragroups than in the intergroup crosses could be due to firstly, the hidden relatedness between some parents considered as being related based on their pedigree data, and secondly because of the presence of some linkage phase between quantitative trait loci (QTLs) and the maternal loci in the maternal and paternal gametic arrays of the intragroup, which results in the positive covariance between GD and F_1 or MPH (Melchinger, 1999), and increases the GD versus F_1 or MPH in the intragroup crosses, respectively.

In both intragroup and intergroup crosses the correlation between GD and F_1 or MPH is expected to increase if (i) QTLs influencing heterosis are closely linked to markers

used in the calculation of GDs; and (ii) markers used to calculate GDs are linked to QTLs. So increasing the marker density alone will not necessarily improve the quality to predict MPH by GD estimates, rather markers must be selected for tight linkage to QTLs affecting heterosis of the target trait in the germplasm under study (Charcosset *et al.,* 1991). However, attempt to identify QTLs-marker associations by using regressing phenotypic values of fixed set of hybrids on the their coded marker genotypes must be regarded with great caution, since Lübberstedt *et al.* (1998) reported that QTLs regions affecting a given trait are not consistent across different germplasm. Results from this study revealed that maximum yields were mainly obtained from intergroup crosses whereas the lowest yields were produced when genetically related lines were crossed. For example, intergroup crosses such as line 9 x line 8 (GD = 0.63), line 1 x line 2 (GD = 0.61) and line 14 x line 13 (GD = 0.57) could produce yields of approximately 9 MT/ha, 7.42 and 6.20 MT/ha, respectively across three different sites, while an intragroup cross such as line 15 x line 10 (GD = 0.30), line 10 x line 4 (GD = 0.32) and line 17 x line 10 (GD = 0.32) produced only approximately 3.15, 2.9 and 2.3 MT/ha in multienvironments (data not shown). This is because an increase in GDs between the two parents will positively increase heterosis for the cross (Cheres *et al*., 2000) or in quantitative genetic theory heterosis is said to be a function of gene frequency differences between the two parents, the level of dominance and epistatic gene effects (Falconer and Mackay, 1996).

According to the quantitative–genetic theory, mid parent heterosis (Falconer and Mackay, 1996) is a linear function of dominace effects and high parent heterosis as a function of both additive and dominance effects. Computations of correlations between GD and GLS-MPH, GD and GLS-HPH in this study showed that there was a tighter association between GD estimated and the HPH than the MPH for GLS resistance (Table 6). Mahn (1977) reported that GLS resistance in maize is controlled by mainly additive genes with narrow sense heritability (h^2) of GLS resistance of 0.33. Narrow sense heritability is a function of additive genes as given by the formula that heritability is the ratio of additive variance over phenotypic variance. Based on this it follows that additive genes contribute to GLS resistance in maize. However, the calculated correlation coefficient between GD and GLS-HPH is greater than the correlation between GD and GLS-MPH. This implies that GLS-HPH mainly

contributes to additive genes for GLS resistance in maize and a small percent by a dominant gene, consistent with other published findings. Lastly, it was also observed that after addition of the absolute values of r(GD, GLS-HPH and GLS-MPH) gives 0.37. This value is also similar to the narrow sense heritability of GLS resistance, which is 0.33. Thus based on this observation it is suggested that the summation of the two correlation coefficients of GLS-HPH and GLS-MPH could potentially be useful in the calculation of narrow sense heritability of GLS resistance in maize.

Finally, it was observed that there was a tighter correlation in intergroup crosses than in the intragroup crosses. Thus, this study therefore recommends and supports that field-testing of particularly intergroup crosses is inevitable in order to confirm these findings. Also, the research strongly supports the idea that tightly linked DNA markers to QTLs which are responsible for F_1 heterosis of the target traits are desperately needed. The identification of such markers from linkage studies could provide accurate relationships between GDs and MPH or F_1 phenotypic data. In this way high yielding intergroup crosses could be easily identified from GD-MPH estimates and would reduce unnecessary field costs like diallel crossing and evaluations of these crosses in multienvironments.

Conclusion

In conclusion, DNA markers such as AFLPs are powerful tools for identification of genetically similar/different genotypes. Marker systems could reveal genetic relationships among breeding materials (and genetic resources) and such markers might assist breeders in the choice of genetically diverse parents for best line combinations to produce maize hybrids of commercial value and monitor the level of genetic diversity in germplasm pools. Finally, heterotic response (and hybrid performance) between these germplasm crosses could be predicted from GDs based on DNA markers and conformations of such predictions need field evaluations especially for intergroup crosses. Intragroup crosses with low GD-MPH could be discarded in order to reduce field costs. Lastly, it is our belief that better F_1 hybrid performance estimations can be achieved from using both molecular and F_1

phenotypic data than using only molecular data like GDs to predict hybrid performances.

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CHAPTER 5

EVALUATION OF HYBRIDS FOR GLS RESISTANCE IN MULTIENVIRONMENTS

Abstract

Maize production is severely hindered by both biotic and abiotic stresses. Gray leaf spot (GLS) disease in maize is caused by the fungus *Cercospora zea maydis*. This disease reduces both yield and grain quality. GLS is currently regarded as one of the most yield limiting diseases of maize worldwide including Tanzania. Chemical and cultural control methods of GLS are either expensive or less effective or both. Also, many exotic hybrids perform poorly in terms of yield and GLS resistance due to poor adaptability. Thus, the Tanzanian Maize Research Program has put more emphasis to breed maize hybrids that are resistant to GLS. Despite this effort, there are still few GLS insensitive hybrids in the country. Therefore, the main aim of this study was to produce more GLS resistant hybrids for commercial use by using resistance breeding. This control strategy is very effective, safe to the environment and inexpensive. Also, the study aimed to increase the farmer's choice of growing different types of GLS insensitive cultivars and to ensure that GLS insensitive hybrids are available all the time in case of GLS hybrid breakdown of resistance. Finally, this research examined the effects of genotype x environment interactions for grain yield, GLS score and other important traits such as kernels/row, ear length, row/ear, etc. This study used 29 moderately/highly GLS resistant parents to produce 225 maize crosses for the farmers. These hybrids were tested across three locations for three seasons, which is a prerequisite to evaluate genotypes for GLS resistance. The locations were chosen to represent all the high GLS disease pressure areas in the Southern Highlands of Tanzania. The experimental design was a 15 x 15 triple lattice. Statistical analyses revealed that hybrids 48, 90 and hybrid 45 had higher stable yields and consistently low GLS ratings across locations. Furthermore, there were many hybrids that had low/high yields exhibiting low GLS scores. These crosses could be used to make other types of maize cultivars such as open pollinated varieties and doubled haploid hybrids. Finally, it was noted that there are potential GLS resistant lines in the SHT and in Population 62 which could be deployed effectively and or might be used in inbred backcrossing breeding programs. The diallel studies revealed that both GCA and SCA were significant for GLS rating indicating the importance of both additive and dominance gene actions although GCA was more important. In general, the GCA effects of inbreds were good indicators for GLS rating and hybrid performance but could not predict the performance of all hybrids. Final selection for best inbred combinations should also be based on good specific combining ability for yield.

Keywords: Environments; GLS, G x E interactions, Pathotype, RCBD.

Introduction

Cultivars grown across locations react differently to environmental factors such as temperature, soil characteristics, wind, rainfall, etc., (Crossa *et al*., 1999). All these variables are collectively known as environments (Allard, 1999). Every factor, which is an element of the environment, has the potential to cause differential performance of cultivars. The differential response of genotypes across environments is called genotype x environment (G x E) interaction (Shi *et al*., 2002; Calderini *et al*., 2001). Interaction effects are a reflection of competing physiological processes, interaction within and between different genetic systems, and interaction between the environment and these processes and their genetic control (Reynold *et al*., 2002; Rao *et al*., 2002). G x E interaction is a major problem when studying quantitative traits because it complicates the interpretation of genetic experiments and makes prediction difficult.

It is a universal practice to test the new varieties across locations and seasons so as to ensure that forthcoming cultivars have stable performances over a range of environments (Annicchiriarico, 1997). Testing environments should adequately represent the environments that cultivars will eventually be grown in or identify environments that are unsuitable for the new varieties (Gunasereka *et al*., 2003; Reynold *et al*., 2002). Testing locations are important to plant breeders for the following reasons: (1) the potential need for unique cultivars adapted to particular areas require an understanding of genotype x location interaction, (2) effective allocation of resources for testing genotypes across locations and years is based on the relative importance of genotype x location, genotype x year and genotype x year x location interactions, (3) the need to develop unique cultivars for optimum performance depends on the interactions of genotype with predictable environmental factors, and (4) lastly, quantification of cultivar's stability of performances involve evaluating genotypes across environments (Rao *et al*., 2002).

In Tanzania, like any other country where GLS is endemic/epidemic, maize yield is significantly reduced by this disease. GLS was reported in Tanzania for the first time during the 1994/95 season. Since then, GLS is threatening food security and nutrition in the country because maize is a staple food for over 60 % of Tanzanians (Lyimo, per. Comm.*). Currently, there are few commercial hybrids that are resistant to GLS.

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In order to address the production needs of the farmers, the Uyole Agricultural Research Institute, Mbeya region, has developed several moderately/highly GLS resistant parents for commercial hybrid production. Thus, this study attempted to use resistance breeding, which is the cheapest, most effective, easy to use and friendly to the environment method of managing GLS. Therefore, the main aim of this study was to produce 225 hybrids and then screen them for yield, GLS resistance, and other desirable morphological traits across locations and years for commercial use. Furthermore, this research aimed to ensure that there is a continuous supply of GLS insensitive hybrids which are genetically different to replace those which may suffer from GLS disease breakdown due to the appearance of new physiological races of the GLS pathotypes.

Materials and methods

Field evaluation of inbred lines and hybrids

Field experiments aimed to evaluate the 225 hybrids for resistance to *Cercospora zea maydis* (Tehon and Daniels, 1925) infection in high GLS disease pressure areas for the purpose of producing hybrids resistant to GLS. The fieldwork was conducted in the Southern highlands of Tanzania, which included different agro-ecological zones namely; Mbeya region, with an altitude between 1500 and 2500 metres above sea level (masl), Rukwa region with an altitude between 1500 and 1800 masl, and finally Iringa region, with an altitude between 1800 and 2500 masl. The experiment was conducted for three years (2002-2004) across three locations. The experimental sites were: Uyole farm in the Mbeya region (located approximately 650 km from Nkundi farm and 250 km from Matanana farm). The Uyole site has a flat topography with black clay loamy soils. Another site was Nkundi farm which is in the Rukwa region. This location is approximately 900 km from Matanana farm. The Nkundi site has a hilly land with red clay soils. The last experimental site was Matanana farm in the Iringa region is also a hilly topography but with reddish-brown clay soils.

Figure 1. Map of Tanzania showing regions (☆) where this study was conducted. Source: http://www.thewhitefathers.org.uk/350map.html (accessed 1/8/2007).

These locations were chosen to provide differences in soil type, vegetation, annual precipitation, temperature, altitude, etc, representing all the maize growing areas in the Southern highlands of Tanzania. There were 225 hybrids produced from 29 moderately and highly GLS resistant parents (using partial diallel crossing design) during the 2000/2001 growing season at Uyole farm, Mbeya region (Table 1). The crosses were produced for three consecutive years. Seeds from these crosses were used for hybrid

evaluations in the following season until the study was completed. The inbred lines used to make crosses were selected based on previous screening tests for GLS resistance at the Uyole research farm (Lyimo per. comm).

Experimental design

The 225 hybrid combinations (210 single crosses, two control hybrids and 13 filler hybrids) were planted using a 15 x 15 triple lattice design. Inbreds were planted adjacent to the hybrids in a randomized complete block design. Planting was done in mid December every year at all the sites. The plot size was a single row plot, 5.1 m long with inter row spacing of 0.75 m and intra row spacing of 0.30 m. Two kernels were planted in each hill, thereafter plants were thinned to one plant per hill, and this resulted in a population size of 44,400 plants/ha. Disease spreader rows of the highly GLS susceptible genotype (Ph3253) (Lyimo, per. comm.) was planted around the experiments and was also planted every ten crosses in both the hybrid block and inbred lines. These rows were used for monitoring disease development with time and as source of inoculum for the hybrids. Two controls were included, a highly resistant hybrid (UH 615) and a highly susceptible hybrid (Ph3253). GLS infected corn residues were used as source of inoculum for the hybrids and inbreds, and were spread on the ground (Gevers and Lake, 1994).

Morphological traits

The morphological traits recorded included; yield, height to ear, ear length, kernels/ear, rows/ear and GLS score, respectively. The grain yield /plot was measured in terms of kilograms/ha at grain moisture content of 12.5 percent. Data on days to 50 % silking was obtained by counting number of days from the day of planting to the day when 50 % of the plants in each plot had silks. Height to ear was measured as the average height in centimetres from the ground level to the first ear in each plot. Ear length was measured as the average length in centimetres of all ears in each plot. Number of rows/ear was obtained by counting the number of rows/ear for all ears in a plot. The average number of

rows/ear was taken as the overall number of rows/ear of that plot. Number of kernels/row was obtained by counting the number of kernels/row for all ears of a plot. The average number of kernels/row was taken as the overall number of kernels/ear of that plot. Gray leaf score was scored from a scale of 1 to 9 with an increment of 0.25. A genotype with 1 score was highly resistant, while 9 was highly susceptible (Donahue *et al*., 1991).

Table 1. Origin, pedigree and heterotic patterns of maize lines used in this study.

No	Line name	Kernel type	GLS rating	Germplasm source	Pedigree	Established heterotic group†
1	K53015213	Flint	3.25	K205 x K230	K530 $S_5$152-1-3	A
2	K5301482	Flint	3.00	K205 x K230	K530 $S_5$1482 (97)-1	A
3	K53014821	Flint	2.25	K205 x K230	K530 $S_5$14821(98)-1	A
4	P629521	Flint	2.75	Population 62	P62 $s_7$95-2-1	A
5	P621111	Flint	2.50	Population 62	P62 S_7 11-1 -1	A/B
6	P627733	Flint	2.00	Population 62	P62 $s_6$77-3Pap3	A
7	P62L50	Flint	2.25	Population 62	P62 $s_6$86-95-1-4	A
8	P62145	Flint	1.50	Population 62	P62s_7 145-95	A/B
9	CML37	Dent	2.25	Population 32	Pop32c4Hc128-1-1-B-H5	A
10	P621621	Flint	1.75	K205 5x K230	K530 $S_5$66-5 (97)-1	A
11	P628495	Flint	2.50	Population 62	P62 $s_6$84-95Blk	A/B
12	P62103	Flint	8.00	Population 62	P62 103-93	A
13	CML11	Dent	3.00	Population 21	Pop21c5hc219-3-2-2-3#7-1B-4-10b	A
14	E50932815	Dent	2.25	E250 x E393	E5093 s_6Pap28-1-5	B
15	P621321	Flint	1.75	Population 62	P62 $s_7$13-2-1	A
16	K375891	Dent	2.50	K337 x K358	K3758 $S_7$9-1-1-1	A
17	P62101	Flint	3.50	Population 62	P62 101-95	A
18	P62148	Flint	2.50	Population 62	P62 148-95	B
19	E5093642	Dent	3.50	E250 x E393	E5093 $s_7$64	B
20	K37581011	Dent	3.25	K337 x K358	K3758 $S_7$10-1-1	A
21	K3758L36	Dent	2.00	K337xK358	K3758s_6L36	B
22	K53015233	Dent		K337 x K358	K530$s_5$152-3-3	A
23	K3758911	Dent		K337 x K358	K3758$s_7$89-1-1	A
24	K37581011	Dent		K337 x K358	K3758$s_7$10-1-1	A
25	K3758111	Dent		K337 x K358	K3758$s_6$1-1-1-1pap	A
26	K37586911	Dent		K337 x K358	K3758$s_6$69-1-1	A
27	K375869111	Dent		K337 x K358	K3758$s_6$9-1-1	A
28	K37588913	Dent		K337 x K358	K3758$s_6$89-1-3	A
29	K3758L36	Dent		K337 x K358	K3758s_6 L36	A

† (Lyimo per.comm).

Statistical Analyses

Statistical analyses of the hybrids evaluated across three locations and for three years (nine environments) necessitated the use of experimental model where a value or performance of any trait measured was a function of the average performance of that trait across all the three years and three locations, which was also dependant on the interaction of different hybrids with the locations in which they were evaluated or by environmental

factors present in the locations such as temperature, rainfall, duration and intensity of sunlight, altitude, relative humidity, soil characteristics (such as soil type, soil texture, soil pH, soil fertility, etc), drought, etc. Furthermore, a performance of a trait was affected by how an individual hybrid interacted with seasonal and/ or climatic variations of weather conditions like temperature, rainfall and its distribution, wind speed and direction, relative humidity, dry spell, etc. Also, the expression of any hybrid trait was dependant on the genotype of that particular hybrid, in other words, the total genetical endowment of the hybrid i.e. some hybrids were genetically short, others were tall, while some were genetically resistant to GLS disease and others did not have any GLS resistant genes. Also, some had moderate resistance/susceptibility to the GLS pathogen and thus, different hybrids responded differently to the GLS pathogen. Interaction effects such as replications within different locations and seasons could have different impact on the performance of traits among the hybrids. The other type of interaction that could affect the individual performance of a trait in a hybrid could be the three way interaction where by three factors of hybrids, locations, and seasons all together interact in a complex way to cause differential performance of traits among the hybrids. The experimental model also included the error term as a variable which could affect the expression of a hybrid trait. The accuracy/inaccuracy of data recorded by the experimenter during the execution process of the experiment could also affect the expression of a trait which is under the error term of the experimental model.

Thus, in an effort to take all these variables into account of the experimental model, this study was represented mathematically as follows:

$Y_{ijkl} = \mu + \tau_i + L_j + S_k + r(L_j \times S_k) + (\tau_i \times S_k) + (\tau_i \times L_j) + (\tau_i \times L_j \times S_k) + \varepsilon_{ijkl}$ where:
Y_{ijkl} = is a trait performance (e.g. yield).
μ = is the average performance of a trait.
τ_i = is the effect of the $_i$th hybrid.
L_j = is the effect of $_j$th location on the performance of the trait.
S_k = is the effect of $_k$th season on the performance of the trait.
$S_k \times L_j$ = is the effect of the $_k$th season in the $_j$th location.
$r(L_j \times S_k)$ = is the effect of a replication in the $_k$th season and $_j$th location.
$\tau_i \times S_k$ = is the effect of the $_i$th hybrid in the $_k$th season.
$\tau_i \times L_j$ = is the effect of the $_i$th hybrid in the $_j$th location.
$\tau_i \times L_j \times S_k$ = is the effect of the $_i$th hybrid in the $_j$th location and in the $_k$th season.
ε_{ijkl} = is the experimental error effect or noise.

Statistical analyses of SAS software, SAS 1999 version 1, 9.3 programme was used for the data analyses.

Mean yields and the correlation coefficients among the phenotypic traits and location correlations

The mean yields and GLS scores for the 225 hybrid combinations at one location with the yields and GLS scores at each of the other locations were used to determine location similarities (Campbell and Kern, 1982). Correlation coefficients for yield and GLS rating between locations were computed using the SAS procedure (SAS, 1999 version 1.9.3) respectively.

Analysis of Variance

Additional statistics of assigning hybrids into yield (Y-axis and GLS scores in X-axis) was used to group together the experimental hybrids into clusters that differed from one another in terms of GLS disease scores and yields respectively. Furthermore, data were combined over locations and analysed as combined series using the general linear model (GLM) procedure (SAS, 1999 version 9.1.3). All effects were considered as random.

Analyses for general combining ability (GCA) and specific combing ability (SCA) for a 21 maize diallel grown at Uyole, Matanana and Nkundi for 3 years at each site.

Data for grain yield and gray leaf spot rating were analysed using Method 4 Model 1 analysis (Eisenhart, 1947; Griffing, 1956). In these analyses it was assumed that all factors were fixed except environments. This is because the lines used were not a random sample. They were highly selected inbred lines. Combining abilities were calculated as defined by Sprague and Tatum (1942).

Results

The phenotypic correlation coefficients (r) of different traits measured for the 225 hybrids across three locations and three years in this study are shown in Table 2.

Table 2. Phenotypic correlation coefficients between traits studied.

	50 % silking	Ear height	Ear length	Rows/ear	Kernels/row	GLS score	Yield
50%silking	-	0.34*	-0.20*	-0.10*	-0.17*	0.11*	-0.36*
Ear height		-	0.32*	0.17*	0.15*	-0.30*	0.29*
Ear length			-	0.07*	0.60**	-0.14*	0.35*
Row/ear				-	0.02^{NS}	-0.12*	0.16*
Kernels/row					-	-0.03*	0.43*
GLS score						-	-0.34*
Grain yield							-

*, ** significant at the 5% and 1% level of probability, respectively.

Table 2 exhibited that grain yield was positively correlated to all traits studied except GLS disease (r = - 0.34) and days to 50 % silking (r = - 0.36). Furthermore, yield was statistically associated with the following traits: kernels/row (r = 0.43), ear length (r = 0.35), ear height (r = 0.29) and the number of rows/ear (r = 0.16). GLS disease score, however, was negatively correlated with traits such as ear height (r = -0.30), ear length (r = -0.14), number of rows/ear (r = -0.12) and number of kernels/row (r = -0.03), but was positively correlated to days 50 % silking (r = 0.11). Also, results showed that the number of kernels/row and ear length recorded the highest correlations (r = 0.60) between any two traits studied. But the number of kernels/row had no association with the number of rows/ear (r = 0.02). Generally, the correlation coefficients among the remaining traits were positive and were between, 0.1 and 0.3.

Phenotypic associations (yield and GLS traits) for all the hybrids across the three locations are exhibited in Table 3.

Table 3. Correlations of grain yield and GLS for 225 hybrids across 3 locations and 3 seasons.

	Uyole site	Matanana site	Nkundi site
Uyole site	-	0.60* (0.67**)	0.71** (0.85**)
Matanana site		-	0.54* (0.77**)
Nkundi site			-

( ) Correlation coefficient for yield
*, ** significant at the 5% and 1% level of probability, respectively.

Phenotypic correlations (Table 3) revealed that the highest correlation coefficient for yield was in the order of: Uyole-Nkundi sites (0.85) > Nkundi-Matanana sites (0.77) > Uyole-Matanana sites (0.67). Furthermore, Table 3 also showed that Nkundi-Uyole sites recorded the highest association for GLS (0.71). However, the lowest association for this disease was exhibited between Nkundi-Matanana (0.54) sites. The GLS correlation coefficient between Matanana-Uyole was 0.60.

Inbred line general combining ability (GCA) effects for gray leaf spot and grain yields from a 21 maize diallel grown across 3 locations (Uyole, Matanana and Nkundi) from 2001 to 2003 in this research is shown in Table 4.

Table 4. Inbred general combining ability effects from a 21 maize diallel grown across 3 locations for 3 years

		GCA effects	
Line no	Inbred name	Gray leaf spot rating	Grain yield (kg/ha)
1	P62145	-0.6	601.8
2	K53015213	0.1	-112.6
3	K375891	-0.1	-16.4
4	K37581011	0.1	605.9
5	K5301482	-0.2	-122.3
6	P627733	-0.3	340.0
7	K3758L36	-0.2	89.0
8	K53014821	-0.1	237.0
9	CML37	0.1	512.1
10	P629521	0.1	119.7
11	CML11	0.2	154.6
12	P621621	-0.5	316.6
13	P621111	0.0	154.0
14	P62L50	0.2	140.5
15	P628495	0.0	416.1
16	P62103	0.8	-405.3
17	P62101	-0.1	-726.6
18	P62148	-0.2	226.6
19	E50932815	0.1	-814.7
20	E5093642	0.0	100.0
21	P621321	-0.4	-900.5
	LSD (0.05)	1.0	1800.0

The inbred line general combining ability effects for gray leaf spot disease ratings and grain yields revealed that lines P62145 (-0.6), P621621 (-05) and P621321 (-0.4) were the three inbreds with the lowest GCA effects for GLS rating. Furthermore, inbred lines K375891 (-0.1), K53014821 (-0.1), P62101 (-0.1), K5301482 (-0.2), K3758L36 (-0.), P62148 (-0.2) and P627733 (-0.3) had GCA effects for GLS rating below zero. Inbred lines P621111, P628495 and E5093642 exhibited GCA effects for GLS rating being 0.0, while lines K53015213, K37581011 and E5093642 showed positive GCA effects (0.1) for GLS rating. Also, lines CML11 and P62L50 had positive GLS effects (0.2). The highest GCA effect for the rating was exhibited by line P62103 (0.8) which was the most susceptible line in this study. The GCA effects for grain yield (kg/ha) was highest for line P621321 (900.0) and lowest for inbred E50932815 (-814.7). The other four inbreds

which recorded the highest GCA effects were the following lines: line K37581011 (605.9), line P62145 (601.8), line CML37 (512.1) and finally line P628495 (416.2). Whereas lines P627733 (-840.0), P62103 (-405.3) and line K5301482 (-122.3) had the lowest GCA effects for grain yield in this research.

The inbred line specific combining ability effects for gray leaf spot rating in this research is revealed in Table 5.

Table 5. Inbred line specific combining ability effects for gray leaf spot rating from a 21 maize line diallel grown at Uyole, Matanana and Nkundi sites for 3 years.

	Line 1	Line 2	Line 3	Line 4	Line 5	Line 6	Line 7	Line 8	Line 9	Line 10	Line 11	Line 12	Line 13	Line 14	Line 15	Line 16	Line 17	Line 18	Line 19	Line 20	Line 21
Line1	-	0.1	0.2	0.1	0.5	0.1	0.4	0.1	-0.3	-0.1	-0.2	0.3	0.0	0.1	0.4	0.2	-0.2	-0.3	0.1	-0.2	0.4
Line2			-0.1	-0.2	0.1	0.3	0.4	0.2	-0.1	-0.2	0.0	0.2	-0.1	0.0	0.1	-0.4	-0.1	0.2	0.0	-0.1	0.2
Line3				0.1	-0.2	0.2	0.0	0.3	-0.3	0.1	0.1	0.1	-0.2	0.1	0.3	0.0	0.3	0.1	-0.2	0.0	-0.1
Line4					0.2	0.3	0.1	-0.3	-0.1	-0.2	0.0	0.1	0.0	0.0	0.0	-0.3	0.2	0.3	-0.1	-0.2	0.4
Line5						0.0	0.2	0.1	0.4	0.1	-0.4	0.1	0.2	0.3	-0.1	-0.2	0.2	0.1	-0.4	0.1	0.0
Line6							0.1	0.2	0.0	0.1	0.3	0.3	-0.1	0.1	0.0	-0.1	0.1	0.3	-0.3	0.3	0.7
Line7								0.2	0.3	0.1	0.0	0.2	0.0	0.0	0.0	0.2	0.1	0.3	-0.2	0.1	0.2
Line8									0.0	0.1	0.2	0.1	0.2	0.4	0.0	-0.3	0.1	0.2	-0.1	0.2	0.1
Line9										-0.2	0.1	0.2	0.3	-0.1	0.2	-0.2	0.0	0.4	0.0	0.0	0.1
Line10											-0.1	0.3	0.1	0.0	0.1	-0.1	0.0	0.2	0.0	0.1	0.2
Line11												0.2	0.1	-0.2	0.2	-0.5	-0.1	0.1	0.2	0.1	0.0
Line12													0.4	0.3	0.3	0.1	0.0	0.2	0.1	0.1	0.2
Line13														-0.1	0.3	0.0	0.2	0.1	0.3	0.2	0.1
Line14															0.0	-0.2	0.1	0.2	-0.1	0.0	0.1
Line15																0.2	0.1	0.3	0.1	0.0	0.2
Line16																	0.0	0.1	0.0	-0.1	0.1
Line17																		0.0	0.3	0.1	0.0
Line18																			0.1	0.1	0.5
Line19																				0.2	0.1
Line20																					0.2
Line21																					

LSD (0.0) = 0.36

Generally, the specific combining ability effects for GLS rating ranged from 0.0 to 0.7. Furthermore, most crosses revealed positive SCA effects for GLS rating as revealed in Table 5. This Table showed that 36 out of 210 hybrids (i.e. 17.1%) in the diallel had SCA

effects of zero, and 82.9 percent had SCA effects for the remaining values. Furthermore, the specific combining ability effects for GLS rating exhibited 35.7 percent of the hybrids had a SCA effect of 0.1 and -0.1. The greatest SCA effect (0.7) for GLS rating was showed by a hybrid line 6 x line 21. This cross, however, was followed by hybrids that had a SCA effect of 0.5. These crosses were line 1 x line 5 and line 21 x line 18. Other hybrids which also exhibited greater SCA effect (0.4) were crosses of line 2 x line 7, line 1 crossed with line 7, line 15 and line 21. Others were crosses of line 5 x line 9, line 4 x line 21, line 14 x line 8, line 18 x line 9 and finally line 13 x line12 respectively. While the lowest SCA effects (-0.5) was exhibited by line 16 x line 11. Crosses which also exhibited low SCA effects (-0.4) included hybrids of line 16 x line 2, line 11 x line 5 etc.

Table 6. Mean squares (MS) for all traits studied for the 225 hybrids across three locations and three seasons.

		MS						
Source of Variation	df	A	B	C	D	E	F	G
1.Locations	2	501708.56**	38345.77**	1991.88**	1329.86**	83.67**	978.20**	2564.03**
2.Seasons	2	194901.66**	4540.08**	2083.68**	3821.39**	69.43**	5953.78**	2383.20**
3.Season x Location	4	57802.46**	10909.34**	312.80**	1053.60**	12.70**	686.56**	220.77**
4. Reps (season x location)	12	4305.81**	179.34**	85.32^{NS}	59.35**	2.15^{NS}	186.27**	121.23^{NS}
5.Hybrids	224	1267.33**	64.24**	9.27**	23.65**	10.87**	114.82**	14.04**
6.Hybrid x Season	448	690.81*	39.11**	4.26**	10.52**	5.80**	55.47**	6.33**
7.Hybrid x Location	448	359.04*	24.24	2.12**	4.89**	1.93^{NS}	26.94^{NS}	2.42**
8.Hybrid x Season x Location	896	365.13*	23.44	2.33**	5.14**	1.91*	26.56**	2.26**
9. Pooled error	3654	319.05	16.49	1.40	3.87	1.75	22.59	1.72

*, ** Significant at the 5 % and 1 % level of probability respectively.
A = Ear height.
B = Days to 50% silking.
C = GLS score.
D = Ear length.
E = Number of rows/ear.

F = Number of kernels/row.
G = Yield (T/ha).

The combined analysis of variance (ANOVA) of all the 225 hybrids revealed statistical differences among the seven traits studied (Table 6). Location effects, however, were found in all the traits studied. The highest location effects were on ear height (s^2 = 501,708.56), followed by days to 50 % silking (s^2 = 38345.77), yield (s^2 = 2564.03), GLS disease (s^2 = 1991.88), ear length (s^2 = 1329.86), number of kernels/row (s^2 = 978.20) and lastly the number of rows/ear (s^2 = 83.67). The seasonal (year) effects on the traits also revealed similar trends as the effects of locations with the highest effects on ear height (s^2 = 194,901.66) and smallest effect on the number of rows/ear (s^2 = 69.43). The number of kernels/row (s^2 = 5953.78) was the second highest trait affected by seasonal weather conditions followed by days to 50 % silking (s^2= 4540.08), ear length (s^2 = 3821.39), yield (s^2 = 2383.20), GLS disease (s^2 = 2083.68) in that order. Furthermore, this research revealed that season by location effects were smallest on the number of rows/ear (s^2 = 12.70) but greater effect on ear height (s^2 = 57802.46), days to 50 % silking (s^2 = 10909.34), ear length (s^2 = 1053.60) and number of kernels/row (s^2 = 686.56). Season by location effects caused variations on GLS disease by a variance of 312.80 and on yield by a variance of 220.77 respectively.

This study also revealed that hybrids exhibited different trait responses. Maximum trait variability among the hybrids was on ear height (s^2 = 1267.33). While moderately trait differences of the crosses were on the number of kernels/row (s^2 = 114.82) and days to 50 % silking (s^2 = 64.24). Ear length (s^2 = 23.65), yield (s^2 = 14.04), number of rows/ear (s^2 = 10.87) and GLS disease (s^2 = 9.27), however, varied lowly among the hybrids. Ear height was highly affected by hybrid x season interactions. The number of kernels /row (s^2 = 55.47) and days to 50% silking (s^2 = 39.11) were moderately affected by the hybrid x season effects. The remaining traits such as GLS disease (s^2 = 4.26) and ear length (s^2 = 10.52) were lowly affected by the hybrids x season interactions. The hybrids across locations varied highly on ear height (s^2 = 359.04) and exhibited low variation on GLS disease (s^2 = 2.12), ear length (s^2 = 4.89) and yield (s^2 = 2.42). Days to 50 % silking, however, was moderately (s^2 = 24.24) affected by the hybrid x location effects. There were no effects of hybrid by location on number of rows/ear and number of kernels/row.

The three way interaction effects (hybrid x location x season) on individual traits exhibited that ear height (s^2 = 365.13) was the mostly affected trait. GLS disease (s^2 = 2.33) and yield (s^2 = 2.26) were equally affected by the three way interaction. The three

way interaction caused lowest variation on number of rows/ear ($s^2 = 1.91$), but days to 50 % silking ($s^2 = 23.44$) and the number of kernels/row ($s^2 = 26.56$) were affected in the same magnitude by the interaction. Ear height ($s^2 = 5.14$) was lowly affected by the three way interaction.

Generally, results of this study showed that ear height was highly affected by locations, seasons, location x seasons, hybrids, hybrid x season, hybrid x location, hybrid x season x location than any other traits studied. But the number of rows/ear was least affected by these sources of variation. GLS disease and yield were also significantly affected by all the sources of variation as well as their interactions.

Table 7. Pairwise *p*-values for interaction effects among location x season on yield and GLS in this study.

	1	2	3	4	5	6	7	8	9	A
1	-	0.0133 *0.9955*	0.0022 *0.0371*	0.0001 *0.4209*	0.0280 *0.0379*	0.0001 *0.0003*	0.0001 *0.0001*	0.0001 *0.0547*	0.0001 *0.1020*	8/72 5/72
2	0.0133 *0.9955*	-	0.0001 *0.0375*	0.0001 *0.4240*	0.6932 *0.0383*	0.0001 *0.0003*	0.0001 *0.0001*	0.0001 *0.0541*	0.0001 *0.1030*	7/72 5/72
3	0.0022 *0.0371*	0.0001 *0.0375*	-	0.0120 *0.1567*	0.0001 *0.9909*	0.0001 *0.0204*	0.0001 *0.0001*	0.1399 *0.0008*	0.0001 *0.5769*	7/72 5/72
4	0.0001 *0.4209*	0.0001 *0.4240*	0.0120 *0.1567*	-	0.0001 *0.1596*	0.0026 *0.0013*	0.0339 *0.0001*	0.1946 *0.0119*	0.0008 *0.03670*	7/72 4/72
5	0.0280 *0.0379*	0.6932 *0.0383*	0.0001 *0.9909*	0.0001 *0.1596*	-	0.0001 *0.0200*	0.0001 *0.0001*	0.0001 *0.0008*	0.0001 *0.5846*	7/72 5/72
6	0.0001 *0.0003*	0.0001 *0.0003*	0.0001 *0.0204*	0.0026 *0.0013*	0.0001 *0.0200*	-	0.1863 *0.0001*	0.0002 *0.0001*	0.5324 *0.0071*	6/72 8/72
7	0.0001 *0.0001*	0.0001 *0.0001*	0.0002 *0.0001*	0.0339 *0.0001*	0.0001 *0.0001*	0.1863 *0.0001*	-	0.0027 *0.0003*	0.0635 *0.0001*	6/72 8/72
8	0.0001 *0.0547*	0.0001 *0.0541*	0.1399 *0.0008*	0.1946 *0.0119*	0.0001 *0.0008*	0.0002 *0.0001*	0.0027 *0.0003*	-	0.0001 *0.0021*	6/72 8/72
9	0.0001 *0.1020*	0.0001 *0.1030*	0.0001 *0.5769*	0.0008 *0.3670*	0.0001 *0.5846*	0.5324 *0.0071*	0.0635 *0.0001*	0.0001 *0.0021*	-	6/72 3/72

The Italicised are *p*-values for GLS respectively.
The 9 Environments or 9 location x seasons are:
1 =Uyole location year 2001season.
2 = Uyole location year 2002 season.
3 = Uyole location year 2003 season.
4 = Matanana location year 2001season.

5 = Matanana location year 2002 season.
6 =Matanana location year 2003 season.
7 =Nkundi location year 2001season.
8 = Nkundi location year 2002 season.
9 = Nkundi location year 2003 season.
A = % overall total significant effect of the season x location.

The pairwise *p*-values comparisons for the season x location interaction effects on yield and GLS are shown in Table 7. Research results revealed that the highest significant season x locations interaction effect (8/72) on maize yield was exhibited at Uyole site in the year 2001. While the lowest location x season interaction effect (6/72) on yield were exhibited at Matanana location in year 2003 and at Nkundi site from year 2001 to year 2003. The total location x season interaction effects on yield in this study was 60/72 (= 83.3 percent). The remaining location x season effects on yield was either 6/72 or 7/72, respectively. The level of gray leaf spot disease was significantly affected by season x location effects (8/72) at Matanana site in year 2003 and at Nkundi location in year the 2002. The lowest season x location effect on GLS disease (3/72) in the study period was at Nkundi in the year 2003. Matanana location in the year 2001 recorded relatively low (4/72) season x location effects on GLS. The other location x season interaction effects exhibited moderately low GLS effects (5/64) such as at Uyole site in all the three years of this experiment, and at Matanana location in the year 2002. The remaining location x season interactions recorded effects on GLS disease (6/72 or 7/72) which was also high. Generally, locations across all the three years of this study caused significant effects on GLS by 51/72 (= 70.8 percent), indicating that GLS disease severity is highly influenced by weather conditions and locations as well**.**

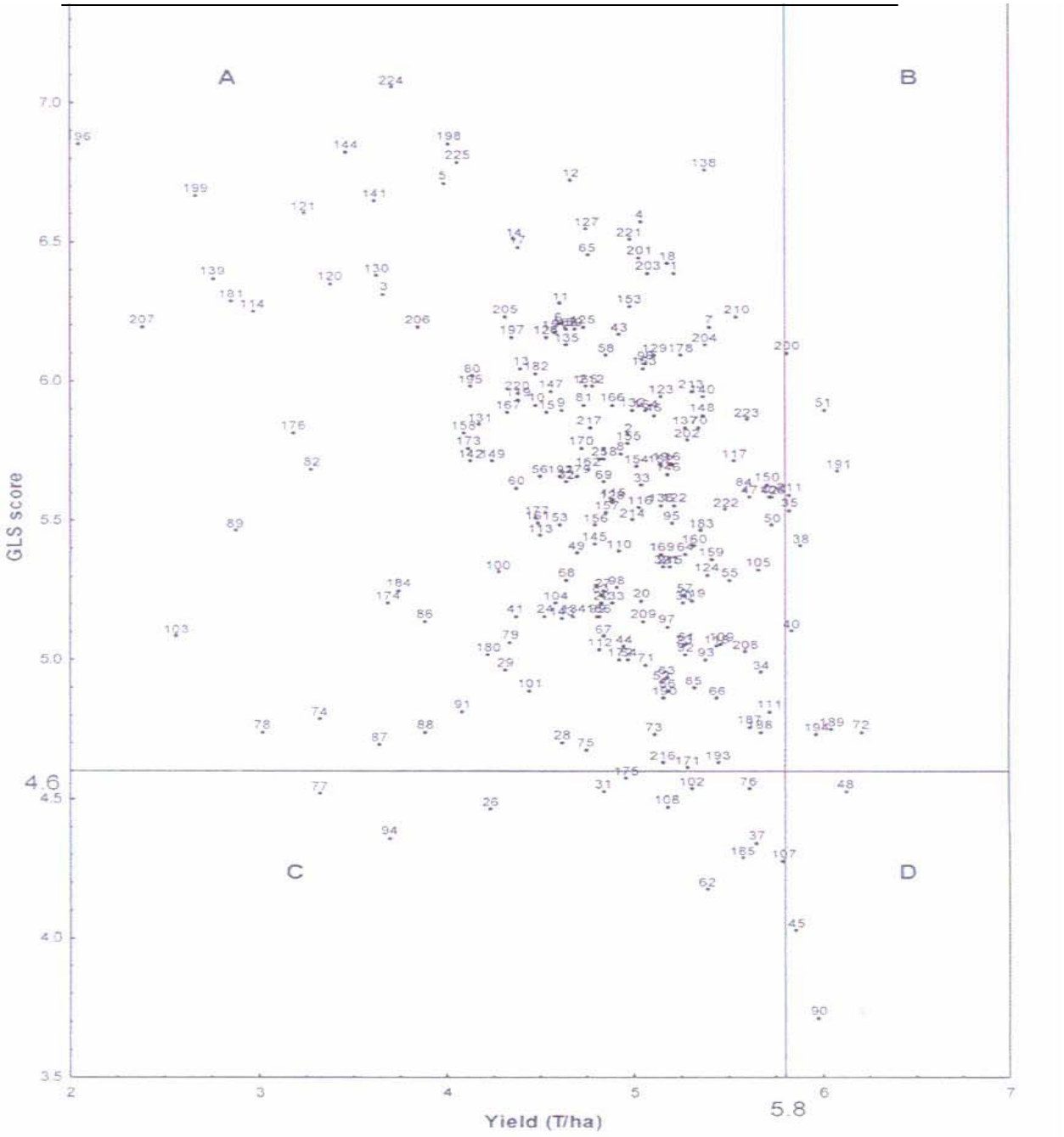

Figure 5. 2. GLS score (Y-axis) and yield (X-axis) of 225 hybrids evaluated across three locations and for three years in this study.

The distribution of hybrids with respect to yield (y-axis) and GLS scores (x-axis) is revealed in figure 2. The figure revealed that few hybrids recorded yields below 4T/ha and few crosses exhibited yields above 6T/ha. Also, few hybrids highly succumbed to GLS (rating of equal or more than 6.5) and few hybrids exhibited high level of GLS

resistance. These hybrids which could be considered as moderately GLS resistant recorded GLS scores between 4.7 and 5.5, respectively. When considering both yield and GLS resistance as the most important agronomical traits for selecting commercial hybrids and using a 4.6 GLS score as a cut off point for GLS disease and 5.8 T/ha as a cut off point for grain yield, then this figure exhibited that hybrids 90, 48, and 45 are in the "D" quadrant. These hybrids could be considered as the top yielding and GLS resistant hybrids in this study. In Table 8 and student's t- test (data not shown) it is revealed that hybrid 90 recorded yield of 5.97, GLS score of 3.71, ranked number 6 (yield), and ranked number 1 (GLS resistance). This hybrid was statistically superior to 130 other experimental hybrids in terms of GLS disease resistance and 200 hybrids in terms of yield. Hybrid number 45 yielded 5.86, GLS score of 4.02, ranked number 2 (GLS resistance), ranked number 9 (yield) and differed with 123 hybrids in terms of GLS resistance and differed with 200 hybrids in yield respectively. Hybrid 48 (yield was 6.12, GLS score was 4.52) significantly differed from 56 other hybrids in terms of GLS sensitivity. Hybrid 48 ranked number 2 in terms of higher yield and ranked the 11.5 th among the GLS resistant hybrids. It differed with 200 hybrids in terms of yield. Furthermore, although not in the "D" quadrant (the highly GLS resistant and top yielding group) were hybrids 72 (yield 6.20), 189 (yield 6.04), 194 (yield 5.96) and 107 (yield 5.78). Their GLS scores were 4.74, 4.75 and 4.27 respectively. Hybrid 72 ranked number 1 (yield) and was number 26th in terms of GLS resistance. Therefore, generally, hybrids 90, 45 and 48 were commercially the best in this study. These were followed by hybrids 72, 189 and 107 which also had superior performances in terms of yield and GLS resistance. Finally, results (Table 8 and or figure 2) revealed that the highest GLS susceptible crosses were hybrids 224 (GLS = 7.05), hybrid 198 (GLS = 6.85), hybrid 196 (GLS = 6.82) and hybrid 144 (GLS = 6.86). While hybrid 90 (GLS = 3.71), hybrid 45 (GLS = 4.02), hybrid 62 (GLS = 4.17) and hybrid 107 (GLS = 4.27) were the most GLS resistant hybrids in this study. Likewise, in terms of grain yield hybrids 196 (yield = 2.04T/ha), hybrid 207 (yield = 2. 38T/ha), hybrid 103 (yield = 2.56T/ha) and hybrid 199 (yield = 2.66T/ha) exhibited lowest yields. While Hybrids numbers 72, 48, 189, 90 and 45 recorded the highest yields.

Table 8. The important agronomic traits of the top 4 hybrids in this study as compared to the lowest/highest trait hybrid performers.

Hybrid	Yield (T/ha)	GLS score	50 % silking	Ear length	Rows/ear	Kernels/row	GLS SD	GLS %CV	Yield SD	Yield %CV	Rank Yield	Rank GLS
72	6.20	4.74	90.22	19.50	14.14	42.25	1.88	30.82	2.43	31.26	1.00	26.00
48	6.12	4.52	93.22	19.02	13.77	40.77	1.71	33.95	1.67	27.35	2.00	11.50
90	5.97	3.71	95.00	19.16	13.04	39.62	1.72	46.41	2.70	45.33	6.00	1.00
45	5.86	4.02	93.22	18.78	14.00	41.25	1.39	34.52	2.05	35.12	9.00	2.00
189	6.04	4.75	92.11	18.80	13.70	41.96	2.35	49.00	2.26	37.44	4	28
194	5.96	4.73	91.96	19.14	13.62	42.14	2.60	55.04	2.86	47.00	7	22.5
107	5.78	4.27	73.92	18.60	13.62	40.70	1.24	29.00	2.20	38.00	14	4
highest	6.20	7.05	97.00	21.21	15.04	44.25	2.58	55.04	2.81	93.60	-	-
Lowest	2.04	3.71	88.04	12.75	11.40	28.55	1.21	26.17	1.37	27.35	-	-

Discussion

Phenotypic associations between different traits studied varied from r = 0.02 to r = 0.60. The correlation coefficients revealed that some traits are highly correlated to each other while others are not. Such information indicates the interrelated stability performance between traits across environments (Snedecor and Cochran, 1967, Steel and Torrie, 1980). Thus significant positive trait correlations can be used to select genotypes with several desirable traits concurrently and hence save time and money. For example, maize yield could be increased by selecting for long ears, more kernels/row, more rows/ear, planting GLS resistant varieties etc. The genetic cause of correlation is chiefly pleiotropy which is simply the property of a gene whereby it affects two or more characters so that if the gene is segregating it causes simultaneous variation in the characters it affects, though linkage is a cause of transient correlation particularly in populations derived from crosses between divergent strains (Falconer and Mackay, 1996). For example, Freymark *et al.*, 1993 reported that chromosomes 2, 4 and 8 had at least one marker with some resistance to *Setosphaeria turcica* at $p = 0.05$, and have also genes for GLS resistance (Bubeck *et*

al., 1993). These genes appear to belong to a resistant gene cluster since *Setosphaeria turcica per se* does not contribute to GLS resistance.

Different yield loses have been reported due to GLS disease. For example, Ward *et al.*, 1997 observed that GLS reduced maize yield between 30 and 60 percent. Donahue *et al.* (1991) documented loss due to GLS to be 10-25 percent in endemic areas and Verma (2001) reported yield losses due to GLS between 28 and 54 percent. Results in this study revealed that yield is statistically reduced by GLS disease ($r = -0.34$). These significant reductions of maize yield due to GLS disease are in line with the findings of Tembo and Pixley, (1999) who reported that GLS became important in the southern and eastern Africa region, where the incidence and severity of GLS epidemics have been increasing. Hence management of GLS disease in Tanzania is inevitable for increased and sustained high maize production.

Research results revealed that Uyole and Nkundi sites had higher associations for both yield and GLS scores (Table 3). This could indicate that these two sites might have similar environmental conditions. These observations are in line with those of Ramon Rea and De Sousa (2002) who reported that sugar cane clones evaluated across six sites had high pairwise correlations ($r > 0.60$) and their highest correlation was between Turbio and Matilde locations (0.82) in Venezuela. These results may help breeders to avoid testing new genotypes in inappropriate environments which have site correlations that are too low with respect to GLS disease. Selection of testing sites should be based on group of locations in which relative genotypic performance is similar and place locations in which relative genotypic performance differs into discrete clusters. One way is to calculate the distance between two environments based on performance data of a common set of genotypes (Bernardo, 2002). Finally, this study revealed that Matanana site had lower associations with other two sites for both grain yield and GLS score. This implies that Matanana location might have some different environmental factors that have greater impact on yield and GLS disease.

The general combing ability effects for gray leaf spot disease rating and grain yield (kg/ha) revealed that inbreds P62145, P621621, and P621321 were the three lines with the lowest GCA effects (shown by negative values) for GLS rating and produced good yields (shown by a positive GCA effects) as revealed in Table 4. These lines with the lowest negative GCA effects for GLS rating are classified as highly GLS resistant inbreds (Donahue *et al*., 1991; Gevers and Lake, 1994). Such lines can be used to introgress GLS resistant QTLs (as donors) to those inbreds that have good combining ability but do

succumb to GLS. Also lines P627733, K5301482, K3758L36 and P62148 exhibited negative GCA effects for GLS rating indicating good level of resistance. Based on the GCA effects for the rating, the latter lines are expected to produce high to moderately GLS resistant hybrids when crossed. Using different maize lines, similar low negative values of GCA effects for GLS rating have been reported in other studies (Donahue *et al*., 1991; Thompson *et al*., 1987; Huff *et al*., 1988; Elwinger *et al*., 1990). Inbred lines with the GCA effects for GLS rating of zero exhibited intermediate GLS resistance. While those lines with highly positive GCA effects (e.g. line P62103) were classified as GLS susceptible lines (Donahue *et al*., 1991). The susceptible lines had low frequency of genes for GLS resistance (Gevers and Lake, 1994). The GCA effects for grain yield in some lines were negative indicating poor combing ability. These research results of GCA effects are similar to those of Donahue *et al*. (1991) who reported that some lines in the diallel crossing exhibited high, intermediate and low GCA effects for GLS rating as well as for grain yields.

Computations of inbred genetic variations based on GCA effects for GLS rating revealed that maize lines in the SHT reacted differently to the GLS pathogen. The GCA effects for GLS rating showed that the resistance to GLS is of additive nature. This is because large GCA effects for GLS rating indicated the presence of additive inheritance (Griffing, 1956; Nakawuka and Adipala, 1997). Furthermore, due to the additive nature of the GLS resistance, inbreds insensitive to GLS in the segregating populations could be selected for during inbreeding process especially from Population 62 of which most of the P62 lines were developed. These lines could then be used to produce GLS resistant hybrids for commercial use.

The SCA effects reflects the deviation of a cross from its expected performance (based on GCA effects) as documented by Olunju *et al*., 1990; Olurunju *et al*., 1992). In this study, however, the hybrid with the greatest SCA effects for GLS rating (line 6 x line 21) revealed a positive SCA effect for GLS rating. This positive effect indicated both of these lines may have the same resistant genes and not able to take the advantage of additive gene action (Donahue *et al*., 1991). Similar to this the SCA effect in fact suggests the presence of dominant or major gene action (Nakawuka *et al*., 1997). Generally the SCA effects for GLS rating of the diallel crosses differed significantly in their GLS disease ratings. The GCA effects for GLS rating (Table 4) of inbreds were accurately reflected in their crosses. For example crosses of line 18 x line 21, line 12 x line 13, line 1 x line 15 etc produced highly GLS resistant hybrids (Table 5). Combinations of line 6 x line 8, line 15 x line 3, line 13 x line 20 etc were moderately resistant. As expected, the crosses of

line 14 x line 11, line 2 x line 16, line 11 x line 16 etc succumbed to GLS. But certain combinations of lines such as line 19 x line 5, line 11 x line 5, line 19 x line 20, line 13 x 15, line 9 x line 13, line 8 x line 14 etc exhibited SCA effects which were significantly better or worse than would be expected on the basis of their general combining ability. Similar findings were reported by Gevers and Lake (1994). Also, Verma (2001) in his findings documented that the performance of hybrids generally agreed with the GCA effects of lines but the relationship was not strong as there were several resistant hybrids whose parents were susceptible with low GCA effects. This implies that GCA effects alone is not sufficient to predict the performance of hybrids (Verma 2001). Tables 4 and 5 showed that both additive and non additive gene actions are important for GLS resistance in maize. The high positive SCA effects for GLS rating which indicates the presence of dominant gene means that such resistance gene can be fixed in commercial hybrid production.

It has been reported that variation in GLS severity among locations is a common phenomenon (Carson *et al.,* 1997) and is most frequently attributed to environmental conditions and tillage practices (Ward *et al.,* 1997). Results in this study showed that some hybrids exhibited mild GLS infections at Uyole and Nkundi, but the same hybrids exhibited higher infection rates at Matanana area. This observation is similar with that of Bubeck *et al.* (1993) who reported that variation in GLS severity may be due to differential sensitivities of maize genotypes to environmental conditions since the quantitative trait loci (QTLs) effects associated with GLS resistance are inconsistent over environments. The other reason could be due to presence of a more virulent GLS pathotype at Matanana location. This observation may need further research to confirm its validity since there have been reports of the existence of sexual reproductive structures like spermagonia and their role, if any, is still debatable (Saghai Maroof *et al.,* 1996; Latterell and Rossi, 1983). Sexual reproduction and mutations could also lead to the occurrence of new GLS pathotype.

It is documented that in variety trial experiments conducted across years and locations, in general, genotype x year effects has larger effects than genotype x environment to these experiments (Simmonds and Smartt, 1999), although both were observed to be nearly equivalent for switchgrass biomass yield (Hopkins *et al*., 1995). The combined analyses of variance in this study revealed a significant location effects, season effects, genotype effects, season x location effects and genotype x environment (hybrid x location, hybrid x seasons and hybrid x location x seasons) interaction effects. Similar results of G x E effects were observed on GLS disease in maize (Bubeck *et al*., 1993; Carson *et al.,* 1997;

Donahue *et al*., 1991). These G x E interactions were due to weather conditions that were favourable for GLS disease development, which varied during the three crop growing seasons of this study (weather data not shown). The 2001/2002 season was highly favourable for the disease especially during the vegetative growth stages up to the "milk stage". During 2002/2003, however, weather conditions at the vegetative stage was dry, but unexpected late rains which were associated with overcast days for two weeks and high relative humidity that came in after flowering stage favoured the development of GLS disease. In contrast, 2003/2004 was hot and dry during the vegetative stage and even after flowering. So the 2003/2004 season was completely unfavourable for GLS development. Thus during the three years of this research, the weather conditions were so variable to cause significant G x E interaction effects as revealed by statistical analyses (Tables 6 and 7). These results are important to breeders in the sense that small weather changes within a season and at any crop stage can cause a significant G x E effects on weather sensitive traits like GLS and yield which depend on the level of GLS infection and the level of genetic resistance of the varieties to the GLS pathogen. Also, results of this research regarding G x E interaction (Table 6 and 7) are in line with the observations reported by Sallah *et al*. (2004) when working on genotype x environment interaction in three maturity groups of maize cultivars documented that genotype x location x year interaction could significantly ($p = 0.01$) affect grain yield in three maturity group of maize. They also observed that genotype x year and genotype x location interaction were also significant in the intermediate and late maturity groups.

Results in Table 8 and figure 2 revealed that there was a continuous distribution of the hybrids tested to GLS disease and to yield responses but with little transgressive segregation. The highest GLS susceptible hybrids were hybrids 224, hybrid 198, hybrid 196 and hybrid 144. While hybrid 90, hybrid 45, hybrid 62 and hybrid 107 were the most GLS resistant hybrids in this study. Likewise, in terms of grain yield the transgressive segregates were the hybrids 196, hybrid 207, hybrid 103 and hybrid 199. The latter hybrids revealed lowest yields because they severely succumbed to the GLS pathogen as they lacked the genetic resistance to this disease. Results of hybrids that are highly susceptible to GLS were also reported by Verma (2001). Hybrids with low GLS resistance revealed low general combining ability effect (for yield) because of non additivity (Verma, 2001, Vivek *et al*., 2001). Furthermore, in Table 8 and figure 2, the hybrids 48, 45, and 90 could be recommended for the release as they exhibited outstanding agronomical performances such as consistently low average GLS scores

across years and locations, ranked best in terms of GLS resistance and out yielded other hybrids. Hybrids such as 72, 189 and 107 also showed commercial agricultural values. These could be regarded as moderately resistant hybrids as they produced higher yields in the presence of the GLS pathogen. They can be released as commercial hybrid varieties well.

Conclusions

Evaluations based on field data in multienvironments provide useful information to determine adaptability and stability of new varieties. It also provides knowledge of the magnitude and cause of environmental effects in maize breeding programs. Statistical analyses in this study revealed that hybrids 48, 45 and 90 were the most resistant and top yielding in this study. Also, hybrids 72, 189 and 107 exhibited higher yields and were moderately resistant to the GLS pathogen. Thus, hybrids 48, 45, 90 and even 72, 189 and 107 could be used for commercial production in the Southern highlands of Tanzania. Alternatively, the last three hybrids could be recombined to make other types of GLS resistant hybrids like modified single crosses, three way crosses, double crosses, open pollinated populations etc in order to create genetically diverse hybrids against GLS breakdown of hybrids which might happen due to appearance of new races of the GLS pathogen. Finally, this study suggests that similar and careful emphases should be placed on sampling locations where by varieties must be grown in an adequate number which are truly representative of the full range of possible environmental conditions. Selection of sites for testing new varieties across environments should be based on locations that are clearly different in terms of altitude, vegetation, temperature, soil type, rainfall etc. One way to achieve this is to calculate the distance between two environments based on the performance data of a common set of genotypes as suggested by Bernardo, (2002). Breeders and agronomists should recommend to farmers new agricultural production alternatives (varieties, plant density etc) that are stable under different environmental conditions so as to minimize the risk of falling below a certain yield level.

Lastly, this research based on GCA and SCA effects for GLS rating, it would be stated that GLS resistance is of additive and non additive gene action. This implies that it is possible to select GLS resistant lines in an inbred line inbreeding program. These

resistant lines could be used as donor parents for GLS resistant genes to those lines that have good combining ability for yield like P62103 but highly susceptible to GLS. Many lines derived from population 62 have high level of GLS resistance. So it is evident that Population 62 has a potential of producing many GLS resistance lines for commercial hybrid production. Also open pollinated varieties such as synthetics/composites/doubled haploid hybrids etc would be developed by using these GLS resistant inbreds. Molecular markers linked to GLS resistant genes can be developed which could be used with phenotypic data to increase the efficacy of selecting GLS resistant maize varieties.

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CHAPTER 6

PRELIMINARY STUDY ON THE DEVELOPMENT OF CAPS MARKERS FOR FUTURE APPLICATION IN MAS STRATEGIES IN THE SOUTHERN HIGHLAND TANZANIAN MAIZE HYBRID BREEDING PROGRAM

Introduction

Most plant traits are quantitative i.e. controlled by many genes (QTLs) together with environmental factors (Babu *et al.,* 2003), however, some traits are controlled by one or few genes (Young *et al.,* 1999). In classical genetic improvement programs selection is carried out based on phenotypes but without knowing which genes are actually selected. The development of molecular markers was therefore greeted with great enthusiasm as it was seen as a major breakthrough promising to overcome this key limitation (Liu and Cordes, 2004). Well-designed studies using genetic markers will undoubtedly accelerate identification of genes linked to quantitative trait loci (QTLs) for marker-assisted selection (Morgante and Salamini, 2003).

It is documented that for most traits, the location and characteristics of genes are unknown. Therefore identification of tightly linked molecular marker is an important step for molecular characterization of plant genes (Yu *et al.,* 1994). The identification of molecular markers linked to specific genes is usually a three-step process: (1) Assessing the mode of inheritance of the plant trait and the molecular marker; (2) Verification of the linkage between the marker and the trait through segregation analysis (Hayes and Goddard, 2003); and (3) Calculation of the recombination fraction and linkage distance (Yu *et al.,* 1994).

Maize crop is severely attacked by gray leaf spot (GLS) disease caused by the fungus *Cercospora zea maydis,* (Tehon and Daniels, 1925). To date, the GLS control methods in use are: field sanitation, crop rotation, chemicals and host resistance. Host resistance, however, is considered the best option for managing GLS as it is environmental friendly, inexpensive and very effective (Bubeck *et al.,* 1993; Saghai-Maroof *et al.,* 1996). Sources of GLS resistance in maize are available in the cultivated crop (Bubeck *et al.,* 1993; Dunkle and Levy, 2000; Gordon *et al.,* 2004) and in the wild maize relatives (Gevers and Lake, 1994). All these genes are important for initiation of marker assisted selection (MAS) in concert with backcrossing in maize breeding programs. It is documented that when wild germplasm is used as a donor parent in backcross breeding, there are problems of linkage drag, whereby an undesirable trait becomes tightly linked with desirable genes

(Peleman and Van der Voort, 2003; Claudio De Giovanni *et al.,* 2003). Thus, the availability of molecular markers linked to the GLS resistant genes and low genetic distance within the cultivated maize germplasm as compared to wild relatives (Rick *et al.*, 1976; Gevers and Lake, 1994) could overcome linkage drag problems, sterility problems associated with cytoplasm and increase the efficacy of MAS. Also since GLS resistance is quantitatively inherited with narrow sense heritability (Mahn, 1977), MAS could be efficiently used to select maize genotypes resistant to GLS. Several published papers such as that of Pratt *et al*. (2003) suggested that it is important to breed for GLS host resistance by using both conventional and molecular marker assisted selection. They viewed it as important components of integrated pest management (IPM) of disease control strategies. Similar studies of using MAS to select GLS resistant hybrids were proposed by Lehmensiek *et al*. (20001), Bubeck *et al*. (1993) Gordon *et al*. (2004) etc. They further added that MAS is able to pyramiding quantitative resistance factors.

In Tanzania, GLS is an important maize disease hindering maize production. In order to control this disease, the Tanzanian Maize Breeding Program produces many hybrids yearly and screen them phenotypically for GLS resistance in multi-environments. However, pitfalls of phenotypic selection are firstly, this type of selection is not very effective for lowly inherited traits like GLS resistance. Secondly, susceptible genotypes can be selected for which have escaped the disease by chance, and thirdly, selections of GLS resistant genotypes using developed molecular markers from different backgrounds become less effective and reliable when used across other backgrounds. Thus, the main aim of the preliminary study was therefore to develop cleaved amplified polymorphic sequence markers (CAPS) putatively linked to GLS resistance that in future, after proper testing in Tanzania, can be used in MAS strategies in the Tanzanian Maize Breeding Program.

Materials and methods

Plant material

The population for molecular markers development linked to GLS resistant genes using Tanzanian germplasm was developed at Uyole Agricultural Research Farm, in the Mbeya Region, Tanzania, for two consecutive seasons, during the 2002 and 2003

rain seasons. In the 2002 rain season, the inbred line, P62145, a highly GLS resistant commercial line was crossed with line P103, a highly GLS susceptible inbred (Lyimo per. comm.*[1]). The resulting $F_{1:2}$ population was selfed to produce an $F_{2:3}$ progeny which was segregating for the GLS resistant genes in the 2003 rain season. When the $F_{2:3}$ plants were about 88 days old, they were morphologically scored for GLS disease on individual plant basis according to Donahue *et al.* (1991) (scale where 1 = no GLS symptoms, with an increment of 0.25, to 9 = highly GLS susceptible). During 2004, the $F_{2:3}$ plants were grown to produce $F_{3:4}$ in the green house of the Pretoria University, South Africa, and DNA were extracted from each plant.

DNA extraction

Genomic DNA was extracted from leaves of each GLS scored $F_{3:4}$ plant as described by Doyle and Doyle (1987). Total genomic DNA was extracted from approximately 100 mg of leaf tissue using 5 % (w/v) Cetyltrimethyl ammonium bromide (CTAB) [0.1 M Tris-HCL (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 1% (w/v) soluble Polyvinylpyrrolidone (PVP) and 0.2 % (v/v) 2-Mercaptoethanol]. Genomic DNA of each plant was precipitated by using either ice-cold isopropanol or 95 % (v/v) ethyl alcohol. The genomic DNA pellets were dissolved in either 100 µl double distilled water or in low TE buffer [10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA] and then stored at -20 °C. Quantification of the genomic DNA was done by using a spectrophotometer.

GLS resistant and susceptible bulks

Two bulks were prepared for GLS marker development and analysis. The resistant bulk (GLSRB), was prepared from the most resistant individuals by pooling together equal DNA concentrations of six $F_{2:3}$ plants (plant numbers 1, 8, 11, 28, 32 and 35) that phenotypically showed no symptoms of GLS disease and were rated as GLS score of one. The susceptible bulk (GLSSB), was prepared in a similar way but in this

[1] *N.G. Lyimo. Uyole Agricultural Research Institute, Box 400 Mbeya, Tanzania.

case the six most susceptible $F_{2:3}$ individuals (plant numbers 67, 69, 105, 106, 108 and 112) were selected on the basis that they highly succumbed to GLS and had a GLS score of nine.

AFLP Analysis for production of bands of interest

Amplified fragment length polymorphism (AFLP) was carried out as described in the IRDye™ Fluorescent AFLP® Kit for large Plant genome analysis with minor modifications. Briefly, a total reaction volume of 12.5µl was used for digestions, and each sample contained 100 ng genomic DNA, 1.0 µl *EcoR*1/*Mse*1 enzyme mix [1.25 U/µl each in 10 mM Tris-HCL (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 µg/ml BSA, 50 % (v/v) glycerol, 0.15 % Triton X-100] and 2.5 µl of 5x reaction buffer [50 mM Tris- HCL (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate]. These were mixed gently, centrifuged briefly, incubated in the water bath at 37 °C for 2 hours and then placed at 70 °C (15 min) to inactivate the restriction enzymes. The second step involved ligation of the adaptor sequences to the restricted DNA fragments.

Ligation was done by mixing 12.0 µl of the adapter mix and T4 DNA ligase [5 U/µl in 10 mM Tris- HCL (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 50 mM KCL, 200 µg/ml BSA, 50 % (v/v) glycerol] to the previous tube. The mixture was centrifuged and incubated at 20 °C for 2 hours. This was followed by performing a 1:10 dilution of the ligation mixture by mixing 10 µl of the mixture and 90 µl of TE buffer [10 mM Tris-HCL (pH 8.0), 1.0 mM EDTA]. Preselective amplification involved mixing 2.5 µl of the diluted (1:10) ligation mixture from the step above, 20 µl AFLP preamplification primer mixture, 2.5 µl of 10x PCR reaction buffer and Taq DNA polymerase (5 U/µl) in a total volume of 25.0 µl. These were mixed gently and then amplified using a thermocycler Gene Amp PCR® System 9700 Model. The preamplification PCR profile was: 20 cycles at 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The preselective products were diluted 1:260 by taking 10 µl of the preamplification product and adding 250 µl of ddH_2O or low TE [10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA]. The selective step had a total reaction volume of 20.0 µl,

with the following consumables: 7.0 µl diluted preamplification DNA template, 2.0 µl 10x PCR buffer, 2.0 µl dNTP (2.5 mM), 1.2 µl $MgCl_2$ (25 mM), 0.8 µl *EcoR*1-ACA (1 mM), 0.5 µl *Mse*1 (6 mM), 0.12 µl Taq polymerase (5 U/µl) and finally 6.38 µl. The PCR reaction for selective was also done in a thermocyler Gene Amp PCR® System 9700 model.

AFLP primer screening and selective amplification

The primers used for pre-amplification and amplification were similar to those described by Vos *et al.* (1995) with *EcoR*1/*Mse*1 extensions ACA/CTG, ACA/CAG, ACA/CCG, ACA/CCC and ACA/CGC. Only two primer combinations (ACA/CGC and ACA/CCG) from the five screened produced polymorphisms between the GLS resistant and GLS susceptible samples, and were thus used in this preliminary study. The *EcoR*1 primers were 5' labelled with infrared dye (1 µM IRDye700 or IRDye 800, LI-COR, Lincoln, NE, USA). The PCR profile for selective amplification was: one cycle of 94 °C for 10 s, followed by 13 cycles of 65 °C for 30 s, with 0.7 °C decrease/cycle. Then there was 23 cycles of 94 °C for 10 s, 56 °C for 30 s and 72 °C for 1 min, with 1 s decrease /cycle. Finally, one cycle of primer elongation at 72 °C for 1 min.

Electrophoresis and excision of fragments

AFLP fragments were resolved in 8 % LongRanger™ polyacrylamide gels the LI-COR IR2 automated DNA analyser (LI-COR, Lincoln, NE, USA) using a 0.4 mm thickness gel which is suitable for fragment cutting. The AFLP gel was scanned using an Odyssey Infrared Imager instrument (LI-COR Biosciences, Lincoln, Ne, USA) in order to facilitate the cutting of polymorphic bands from the gel. The polymorphic fragments present in the resistant parent and resistant bulk but absent in the susceptible parent and susceptible bulk were excised for cloning. All the exercised fragments were "squashed" in 50 µl low TE [10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA] or ddH_2O and left at 4 °C for a week to facilitate elution of DNA from the gel. Thereafter the samples were centrifuged at 1200 rpm for 5 min and recovered on agarose gel before re-amplification of fragments using the AFLP primer specific to

the fragment. After amplification, each AFLP fragment was verified for purity on a 3 % agarose gel containing ethidium bromide (0.5 μg/ml) and visualised under UV light. Each band on the agarose was excised and recovered with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The excised and purified fragments were cloned into pGEM®- T Easy Vector (Promega) for transformation to competent JM109 *E. coli* cells according to the manufacturer's instructions. The competent cells were prepared adopting the Hanahan (1985) method. The presence of insertions was assessed in a restriction digestion of plasmids following an alkaline lysis preparation (QIAprep 8 mini prep Kit Qiagen) and the sizes of the positively cloned fragments were verified using colony PCR (Gussouw and Clarkson, 1989). The amplicons were subjected to cycle sequencing of both strands using the BigDye™ Dye Terminator Kit (Perkin-Elmer, USA) and the SP6 (5'-ATTCTATAGTGTCACCTAAAT-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primer sets (Promega, USA). Sequencing of clones was done by Macrogen Biotechnology Company, USA.

Primer Design and synthesis

The obtained sequences were subjected to homology searching using BLASTNn and BLASTx (http://www.ncbi.nlm.nih.gov or for further information please see Altschul *et al.*, 1997), and multiple sequence alignment was conducted using CLUSTALW software (http://www.ebi.ac.uk/clustalw). Primer design was done using the obtained sequence information and the Primer 3 software package (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Results

Five AFLP primer combinations were used in Chapter 3 for the prediction of best line combiners and heterosis in Tanzania maize breeding lines. From the initial screening of the breeding lines, primer *EcoR*1-ACA+*Mse*1-CGC had a marker index (MI) of 16.5 and *EcoR*1-ACA+*Mse*1-CCG had a MI of 20.4 (Table 3.3). Two of these, *EcoR*1-ACA+*Mse*1-CGC and *EcoR*1-ACA+*Mse*1-CCG were utilized in the development of putative markers, and screened against the DNA from GLS resistant

parents and GLS resistant bulk and the GLS susceptible parents and GLS susceptible bulk (Figure 6.1).

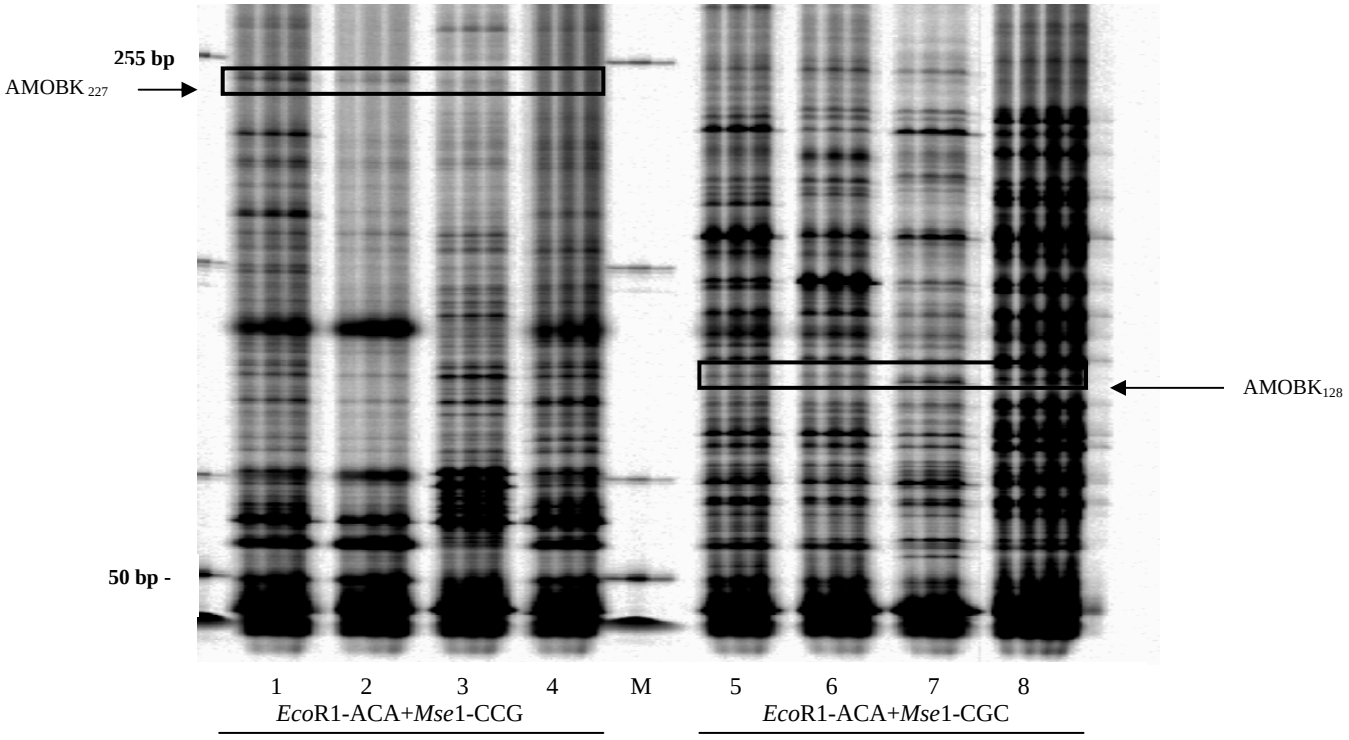

Figure 6. 1. A part of the AFLP gel obtained after AFLP analysis of the susceptible and resistant maize DNA with primer combinations *Eco*R1-ACA+*Mse*1-CGC and *Eco*R1-ACA+*Mse*1-CCG. Where 1 and 5 = GLS resistant parent; 2 and 6 = GLS resistant bulk; 3 and 7 = GLS susceptible parent; 4 and 8 = GLS susceptible bulk; and M = 100 bp marker. Also indicated are two of the polymorphic fragments that were excised, cloned and sequenced characterized, i.e. $AMOBK_{227}$ (227 bp in size) and $AMOBK_{128}$ (128 bp in size).

After analysis of the AFLP profiles, 15 polymorphic bands were obtained which discerned between the resistant and susceptible GLS bulks (Figure 6.1). Of these, four bands were putatively linked in repulsion phase to GLS resistance, while 11 fragments were putatively linked in coupling phase. AFLP primer combination *EcoR*1-ACA+*Mse*1-CGC resulted in polymorphic fragments of approximate sizes of 452 bp and 260 bp in coupling phase, while 190 bp and 260 bp were in repulsion phase. Furthermore, two polymorphic bands in repulsion phase (i.e., sizes 128 bp and 174 bp) were obtained with primer combination *EcoR*1-ACA+*Mse*1-CCG and nine

polymorphic fragments (i.e., sizes 373 bp, 316 bp, 276 bp, 227 bp, 219 bp, 214 bp, 208 bp, 82 bp and 78 bp) were linked in coupling phase (Figure 6.2).

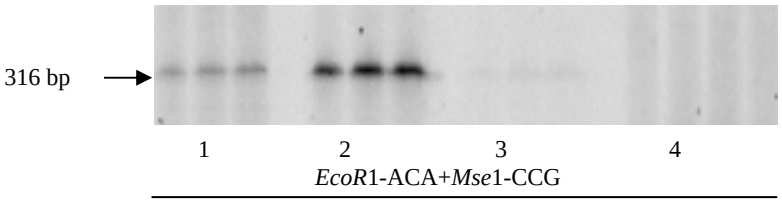

Figure 6. 2. An enlarged section illustrating the polymorphic fragment $AMOBK_{316}$ that discerns between the GLS resistant parent and GLS resistant bulk *versus* GLS susceptible parent and GLS susceptible bulk after analysis using *EcoR*1-ACA+*Mse*1-CCG primer combination. Where 1 = GLS resistant parent; 2 = GLS resistant bulk; 3 = GLS susceptible parent and 4 = GLS susceptible bulk.

Of these putatively GLS linked markers, 15 fragments were excised from the polyacrylamide gels after scanning with the Odyssey Infrared Imager. These were purified, cloned and sequenced. After removal of the vector sequences, the putative sequence annotation and alignment followed. Ten of these sequences were omitted due to poor sequence quality or sequence length making them uninformative (not shown). Surprisingly, the remaining five sequences all showed significant homology to a partial 18S rRNA gene (E-value of $2e^{-69}$) and a partial ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene region (E-value of $2e^{-67}$) of an uncultured soil fungus when using BLASTn (not shown), but to the hypothetical protein 3 from *Microplitis demolitor* bracovirus (E-value of $7e^{-14}$) when using the BLASTx search (Figure 6.3).

```
Query  270  QETAMTMITPSYLGDTIEYSSYASNALGALPYGRPAGGREFTSDYLG----DTIEY  115
            QE AM MI PSYLG  IEYSSYAS ALGALPYGRPAGGREF SD+L     DT EY
Sbjct  37   QEPAMXMIPPSYLGAXIEYSSYASXALGALPYGRPAGGREFXSDFLQMPFLDTEEY  92
```

Figure 6. 3. Clone AMOBK276 exhibiting significant homology to the hypothetical protein 3 from *Microplitis demolitor* bracovirus (E-value of $7e^{-14}$) when using BLASTx search.

The sequences were then aligned using multiple alignment software (Figure 6.4).

```
CLUSTAL W (1.83) multiple sequence alignment

AMOBK227          ------------------------------------------------------------
AMOBK219          ------------------------------------------------------------
AMOBK452          GGGATAAACNTGGATGCCATTGGCGATTGAGCCGACGTCGCATGCTCCCGGCCGCCATGG 60
AMOBK316          ------------------------------------------------------------
AMOBK276          ------------------------------------------------------------

AMOBK227          ------------------------------------------------------------
AMOBK219          ------------------------------------------------------------
AMOBK452          CGGCCGCGGGAATTCGATTGATGAGTCCTGAGTAACCGAGAAGAAAATCATCAGGAACCA 120
AMOBK316          ------------------------------------------------------------
AMOBK276          ------------------------------------------------------------

AMOBK227          ------------------------------------------------------------
AMOBK219          ------------------------------------------------------------
AMOBK452          CACAGCCAATGCCAAAGCAAAGGTCGTGTTGATATCTGAAGTTGGGACGATACGCCATTT 180
AMOBK316          --------GGGGGAGTATGAGNAANNCGATATGAAGGGGTATTGGGTAACCAA------C 46
AMOBK276          -------------------ATAAACGCG-TAGATAATGCGATTGGGCCGACG-------T 33

AMOBK227          ----------------------------------AAAAGGGCATACATGTGTGATGAGCC 26
AMOBK219          ----------------------------------AAAAAGCGATACTTGGGCGATGAGCC 26
AMOBK452          CGTATGTTCGCCGCCGATTACGGAAGCCACATAGGCCACATGATCTACCGGCAGGAAGTC 240
AMOBK316          CGCATGCGTTCAGCCG-CCGTGGCGGCCGACCGCAGGCTCGACCATATAGGGAGAGCTCC 105
AMOBK276          CGCATGCTCCCGGCCG-CCATGGCGGCCGCCTGCAGG-TCGACCATAT-GGGAGAGCTCC 90
                                                                 *       *

AMOBK227          GA-CGTGCATGCTCCCGGCCGCCATG-----GCGGCCGC--GGGAATTCGATATCA-TA- 76
AMOBK219          GA-CGTGCATGCTCCCGGCCGCCATG-----GCGGCCGC--GGGAATTCGATATCACTA- 77
AMOBK452          CATCGCGTTCATCACCAGTACCCACACGAATACGGTTACTCAGGACTCATCAATCACTA- 299
AMOBK316          CAACGCAGTTGGATGCA-TAGCTTTGAGTATTCTATAGTG-TCACCTAAATAATCACTAT 163
AMOBK276          CAACGC-GTTGGATGCA-TAGCTT-GAGTATTCTATAGTG-TCACCTAAATAATCACTA- 145
                    * **          *     *          *             *     **** **

AMOBK227          GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA 136
AMOBK219          GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA 137
AMOBK452          GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA 359
AMOBK316          GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA 223
AMOBK276          GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCA 205
                  ************************************************************

AMOBK227          TAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC-TGT 195
AMOBK219          TAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC-TGT 196
AMOBK452          TAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGTGTGC 419
AMOBK316          TAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC-TGT 282
AMOBK276          TAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGC-TGT 264
                  ***************************************************  **

AMOBK227          -TTCCTGA-------------- 202
AMOBK219          -TTCCTGAT------------- 204
AMOBK452          GTTCCTGAA------------- 428
AMOBK316          -TTCCTGATTTAAAGACGATTT 303
AMOBK276          -TTCCTGACAGA---------- 275
                   *******
```

Figure 6. 4. Nucleic acid alignment of five cloned fragments putatively linked to GLS resistance. Asterisks (*) represent regions of sequence consensus, while gap (-) represents openings. Forward and reverse primers are indicated in red bold text.

The multiple sequence alignment showed significant sequence consensus (i.e., 115 bp) between the different cloned fragments ($AMOBK_{219}$, $AMOBK_{227}$, $AMOBK_{276}$, $AMOBK_{316}$ and $AMOBK_{452}$) in the region with significant homology to the partial 18S rRNA gene (E-value of $2e^{-69}$) and ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene regions (E-value of $2e^{-67}$) of an uncultured soil fungus. The reason for this is still puzzling, since the clones were all different in size when verified using colony PCR technology (not shown).

Primers were then designed using Primer 3 software (Figure 6.4). Since all the targeted fragments are putatively linked to GLS resistance in coupling phase, two strategies were followed during primer design, namely specific primers (i.e., outside the consensus region) and generic primers (i.e., targeting the consensus region). The primers will in future be tested on Tanzanian populations presently in preparation.

To conclude, although the predictions for these primers to be "useful" as GLS linked markers are low, since the excised fragments shared a surprisingly high sequence identity, they will still be tested once the populations for testing are available. However, the search for more putative markers is ongoing, but due to time constraints the results will not be included in the thesis document.

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CHAPTER 7

CONCLUSION

Maize is the staple food for more that 60 % of Tanzanians, but its production and productivity are highly reduced by gray leaf spot disease especially in the Southern highlands of Tanzania. This disease significantly reduces grain yield, kernel quality and silage quality. In order to manage this disease, however, different GLS control strategies such as the use of fungicides, field sanitation, crop rotation, removal of field debris and combination of methods are widely applied. But these control measures are either expensive or biologically unfriendly to the environment or less effective. Furthermore, adoption of exotic maize varieties usually fail due to poor adaptation. Thus, the Tanzanian maize research has put more efforts to breed maize hybrid varieties that are resistant to GLS by using conventional breeding methods which may not be always very effective for traits like GLS resistance that is poorly to moderately inherited. Despite all these efforts, there are still few commercial hybrids which are insensitive to GLS pathogen in Tanzania.

This study, therefore, aimed to produce more GLS resistant hybrids for commercial use by integrating molecular marker technology and conventional resistance breeding which is much safer to the environment, more effective than other control methods and an inexpensive strategy of GLS control. Also, the study aimed to increase the farmers choice of growing different types of GLS insensitive hybrids and ensure a constant supply of GLS resistant hybrids in case of GLS hybrid breakdown of resistance. Furthermore, no molecular data on maize is available for Tanzanian maize cultivars which could assist plant breeders to choose inbreds with regard to carrying combining ability in the production of commercial maize hybrid varieties. Thus, the identification of best inbred combiners is still a major challenge to maize breeders in Tanzania. Many breeding strategies such as crossing parents from different heterotic groups, pedigrees, use of tester lines, etc have been used extensively. Lack of progress in breeding for resistance to GLS has been attributed to the limited effectiveness of phenotype-based selection due to the impact of environmental factors. Hence there is a need to combine GLS resistant genes and high yielding traits in hybrids. Complementation of molecular marker and phenotypic selections could therefore increase the efficiency of breeding maize cultivars resistant to GLS. This study assessed the genetic diversity of highly/moderately GLS resistant

inbreds of Tanzanian germplasm and then predicts the potential of these parents to produce high yielding GLS resistant hybrids. The assessment of genetic variation aimed at testing the genetic variation of the Tanzanian inbreds as a way to ensure against genetic erosion of the present gene pools.

In this study the efficacy of AFLP marker system for grouping inbred lines into genetically similar clusters was assessed. AFLP fingerprinting of genotypes was complemented/supplemented with an investigation which aimed to study the associations between AFLP based genetic distances and F_1 morphological data that included many agronomically important traits like 50 % silking, ear length, rows/ear, kernels/row yield and GLS ratings. Furthermore, this study aimed to develop cleaved amplified polymorphic CAPS marker bands putatively linked to GLS resistant genes which in future can be tested and applied in marker-assisted selection (MAS) to identify high yielding GLS resistant hybrids in an efficient way and/or in marker based backcrossing programs to develop parents in a shorter period of time.

DNA fingerprinting of the 21 inbred lines using 5 AFLP primer combinations detected a total of 259 AFLP marker bands of which 83.2 % were polymorphic and 16.8 % were monomorphic. The average genetic distance (GD) of all the 21 lines was 25.5. The GD of pair wise line crosses varied from a minimum of 0.14 to a maximum of approximately 0.5. Theoretically, the GDs of 0.5 crosses are expected to produce high yielding commercial hybrids, but such crosses will require field observations to confirm their validity in GLS “hot spot” studied zone. The study results exhibited that the AFLP marker as a fingerprinting tool showed higher r(GD, f) than the correlations recorded with SSR and RFLP in previous studies which also proves its superiority and power on assaying a lot of genetic loci.

The UPGMA dendrogram grouped together the assayed lines into three main clusters and four outliers. The AFLP results effectively grouped the lines according to the established heterotic groups but with very minor discrepancies since the established heterotic groupings are all based on morphological data. Morphological data are not capable of

identifying those traits that are masked/unadapted/recessive. Results also revealed that the inheritance of resistance to GLS is low.

In addition to the AFLP study, a pair wise genetic analysis of the inbred lines with their F_1 morphological data revealed the genetic distance of intergroup crosses was much higher than the mean genetic distance of intragroup crosses. This implied that higher yielding hybrids are predicted from intergroup (i.e. between populations) crosses than from intragroup crosses. Results also identified crosses between heterotic groups such as those which exhibited high pair wise GD of more than 0.40. These crosses are line 21 (P62145) crossed with the following lines: line 13 (K53015213), line 14 (K37581011), line 16 (CML37) and line 18 (P621111), as well as crosses of line 13 (K53015213**) x** line 14 (K37581011). These crosses with high pair wise GDs are predicted to produce higher yields due to their genetic dissimilarities, and line P62145 showed the best general combining ability (GCA) in this study.

A genotype x environment analysis (G x E) of the 225 highly/moderately GLS resistant hybrids evaluated in multi-environments revealed that hybrids 90, 45, and 48 were the top yielding and consistently exhibited lowest GLS susceptibility across locations and years. Also hybrids 72, 189 and 107 recorded higher yields and good GLS tolerance. Finally, in a preliminary study 5 cleaved amplified polymorphic sequence (CAPS) markers putatively linked to GLS resistant genes in the studied maize population was developed.

In conclusion, the AFLP DNA fingerprinting of the Tanzanian lines have proved to be powerful tools, highly reproducible, could assay a lot of genetic loci and can be effectively used for characterization of lines and clustering of germplasm according to their genetic similarities. Similar AFLPs studies could be conducted in other Tanzanian maize breeding programs for the protection of breeders' rights of the released varieties and/ or elite commercial inbred lines and for the prediction of best inbred combinations for commercial hybrids use. Intergroup crosses especially with high GD-MPH associations should be the main target for the production of commercial hybrid varieties

as they always show high level of heterosis which is a function of crossing genetically dissimilar lines. Intragroup crosses generally exhibit low pair wise GD-MPH associations. Hence intragroup hybrids are always not suitable for commercial hybrid production. They may also produce hybrids that are more prone to both biotic and abiotic stresses and also may suffer more from the deleterious effects of inbreeding depression and degeneration in comparison to intergroup crosses. However, the intragroup crosses could be useful in the production of modified single crosses, three way crosses and single hybrid seed production if the inbreds used in the crosses exhibit a certain level of heterosis or complement/supplement each other for the traits that are agronomically important for the resulting hybrids. Furthermore, some intragroup hybrids might also be used to make silage as they can exhibit more vegetative growth as the main sink instead of ears.

Finally, the G x E study results showed that GLS disease is highly influenced by both weather factors and locations and thus it is important that new varieties should be tested in different weather conditions as well as locations. Highly GLS resistant with high level of general and specific combing ability should be used. Also testing locations should be truly representative of all the areas that are agro-ecologically different in terms altitude, annual precipitation, soil type, temperature, with regard to disease occurrence and be done over seasons until significant year effects in terms of GLS severity and incidence are revealed. Lastly, characterization of the GLS pathogen is imperative since information on virulence of isolates is needed for long term management strategies against the pathogen.

ANNEXURE

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The prediction of best line combiners and heterosis in Tanzanian maize breeding lines through the use of Amplified fragment length polymorphism (AFLPs)

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Amplified fragment length polymorphism DNA markers have been used to assist plant breeders in the choice of maize parents for commercial hybrid production. However, maize yield in Tanzania is significantly reduced by gray leaf spot (GLS) disease, which is now regarded as the most yield limiting disease of maize worldwide. Thus combining GLS resistance genes and high yielding traits in hybrids is an important breeding strategy. The main aim of this study was to assess the genetic diversity of Tanzanian germplasm and to predict the potential of these inbreds in producing high yielding GLS resistant hybrids. Furthermore, the potential of such data to predict the best line combiners was investigated. This research used AFLP-DNA fingerprinting data from 21 moderately and highly GLS resistant maize inbreds. Results revealed that the genetic distance (GD) of some intergroup crosses were as high as 0.5. Theoretically, these intergroup hybrids with high GD could potentially produce high yielding GLS resistant hybrids. However, such hybrids would require field-testing in order to confirm these observations. Finally the results revealed high *f* values consistent with other reports in maize. In summary these results also corroborates the usefulness of AFLP in genetic diversity studies of germplasm, prediction of best line combiners and high heterosis level for commercial maize hybrid production.

Keywords: Coancentry coefficient (*f*), genetic distance, inbreds, interpopulation, intrapopulation,

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Introduction

Development of inbred lines, followed by their assignment into appropriate heterotic groups and finally planning the desired crosses for superior hybrid production requires adequate knowledge of genetic diversity (Hallauer *et al.*, 1988). This applies particularly to maize hybrid breeding where recognition and exploitation of heterotic patterns between genetically dissimilar sources of germplasm are important for success and also when it comes to proprietary issues and plant variety protection. Genetic diversity in cereal collections is critical in finding new alleles that will improve yields to fight world hunger (Warburton *et al.*, 2002). Even unadapted parents with poor phenotypes can contribute favorable alleles to their progeny when these adapted genotypes are placed in unfavorable backgrounds. Therefore screening based on phenotypes may miss much of the favorable variation, and thus new allelic variation may be identified by means of markers and can be complimented with phenotypes (Tanksley & McCouch, 1997).

Various DNA based fingerprinting techniques are available for genetic variability studies (Liu & Cordes, 2004; Botha *et al.*, 2004; Kosman & Leonard, 2005), and these provide powerful tools in the identification of genetically similar/dissimilar germplasm. In maize, RFLP have been used quite extensively for this purpose (Melchinger, 1993). However, RFLP assays are labour intensive and time consuming and therefore are increasingly substituted by other techniques based on the polymerase chain reaction such as AFLP and simple sequence repeats (SSR). AFLP are genomic fragments detected after selective amplification (Vos *et al.*, 1995). The major advantages of AFLP markers compared with markers like RFLP and SSR is the generation of multiple marker bands in a single assay without prior knowledge of sequence and are highly reproducible (Myburg *et al.*, 2001; Botha *et al.*, 2004). The usefulness of AFLP markers for genetic diversity studies has been demonstrated in many crops (Mackill *et al.*, 1996; Powell *et al.*, 1996; Pejic *et al.*, 1998).

Many maize breeding programs in Africa, for example, in Tanzania, has not yet been studied at a molecular level in order to know the extent of genetic diversity which are present in their working gene pools. Such information are important for biotic/abiotic resistance breeding and for the production of varieties/hybrids which are higher yielders and resistant to different stresses. Furthermore, such information could also help to show the composition of genetic variation in these materials as a strategy to identify sources of biotic and abiotic stress resistance. Finally, studies of this nature could help to suggest appropriate measures that should be taken to sustain the existing genetic variation and to guard against depletion of the present gene pools. In this study, the genetic distance of inbreds using AFLPs was determined. The intended use of the acquired information was to assess the level of genetic diversity of the Tanzanian maize gene pools, to test the usefulness of AFLP to determine best line combiners for increased hybrid heterosis, and to predict the potential of these inbreds to produce high yielding GLS resistant hybrids for commercial use.

Material and methods

Plant material

Twenty-one inbred lines were selected from the Southern highlands (1500 and 2500 metres above sea level) of Tanza-

nia (SHT) from the maize breeding program located in the Mbeya region (Table 1). This region produces more than 50% of the country's annual maize output (Personal comm., Lyimo, N.G., Agricultural Research Institute, Mbeya, Tanzania). These inbred lines were analysed using AFLP analysis. The selection of inbred lines was based on their previous performance in a screening test for GLS resistance (Lyimo, N.G.). Three seeds of each inbred line were sown in a plastic pot containing approximately 1.5 kg mixture of four parts sterilized clay soil and one part sandy soil. Approximately 120 grams of a compound fertilizer of N: P: K (2: 3: 2) was applied to each pot. This pot trial was conducted at the University of Pretoria Experimental farm during the 2000/2001-rain season. At the three to four leaf stage (when plants were about two weeks old), the youngest fresh leaves of each line were harvested and immediately put on ice for DNA extraction.

Table 1 Origin, pedigree and heterotic groups of maize lines used in this study.

No	Name of a line	Kernel type	Source of germplasm	Pedigree	Established † heterotic group
1	K53015213	Flint	K205 x K230	K530 $S_5$152-1-3	A
2	K5301482	Flint	K205 x K230	K530 $S_5$1482 (97)-1	A
3	K53014821	Flint	K205 x K230	K530 $S_5$14821(98)-1	A
4	P629521	Flint	Population 62	P62 s_7-95-2-1	A
5	P621111	Flint	Population 62	P62 S_7 11-1 –1	A/B
6	P627733	Flint	Population 62	P62 $s_6$77-3Pap3	A
7	P62L50	Flint	Population 62	P62 $s_6$86-95-1-4	A
8	P62145	Flint	Population 62	P62$s_6$145-95	A/B
9	CML37	Dent	Population 32	Pop32c4Hc128-1-1-B-H5	A
10	P621621	Flint	K205 x K230	K530 $S_5$66-5 (97)-1	A
11	P628495	Flint	Population 62	P62 $s_6$84-95Blk	A/B
12	P62103	Flint	Population 62	P62 103-93	A
13	CML11	Dent	Population 21	Pop21c5hc219-3-2-2-3#7-1B-4-10b	A
14	E50932815	Dent	E250 x E393	E5093 s_6Pap28-1-5	B
15	P621321	Flint	Population 62	P62 $s_7$13-2-1	A
16	K375891	Dent	K337 x K358	K3758 $S_7$9-1-1-1	A
17	P62 101	Flint	Population 62	P62 101-95	A
19	P62148	Flint	Population 62	P62 148-95	B
20	E5093642	Dent	E250 x E393	E5093 $s_7$64	B
21	K37581011	Dent	K337 x K358	K3758 $S_7$10-1-1	A

†(Lyimo per. comm)

DNA extraction

Genomic DNA was extracted from leaves of each of the lines as described by Doyle & Doyle (1987). Total genomic DNA was extracted from approximately 100 mg of leaf tissue using 5% (w/v) Cetyltrimethyl ammonium bromide (CTAB) [0.1M Tris-HCL (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 1 % (w/v) soluble Polyvinylpyrrolidone (PVP) and 0.2 % (v/v) 2-Mercaptoethanol). Genomic DNA of each inbred was precipitated by using either ice-cold isopropanol or 95 % (v/v) ethyl alcohol. The genomic DNA pellets were dissolved in either 100 µl double distilled water or in low TE buffer [10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA] and then stored at –20 C. Quantification of the genomic DNA was done spectrophotometically.

AFLP analysis

AFLP analysis was performed according to the protocol of Vos *et al.* (1995). Genomic DNA of the maize inbred lines (approximately 100mg) was digested with restriction enzymes *EcoR*1 and *Mse*1. In a second step the following adaptor sequences were ligated to the restricted DNA fragments:

*EcoR*1: 5'-CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA-5'

*Mse*1: 5'-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5'

The primers used for pre-amplification and amplification were similar to those described by Vos *et al.* (1995) with *EcoR*1/*Mse*1 extensions ACA/CTG, ACA/CAG, ACA/CCG, ACA/CCC and ACA/CGC. The five best primer combinations out of 10 screened were chosen for the final selective amplification. *EcoR*1 primers were 5' labelled with infrared dye (1 µM IRDye700 or IRDye 800, LI-COR, Lincoln, NE, USA). The PCR cycle for selective amplification was as follows: one cycle at 94 C for 10s, followed by 13 cycles at 65 C for 30s, with a 0.7 C decrease/cycle. Followed by 23 cycles at 94 C for 10s, 56 C for 30s and 72 C for 1 min, with a 1s decrease /cycle. Finally, one cycle of primer elongation at 72 C for 1 min.

Electrophoresis and image analysis

AFLP fragments were resolved in 8 % LongRanger^{TM} polyacrylamide gels using the LI-COR IR2 automated DNA analyser (LI-COR, Lincoln, NE, USA). The gel images were scored in a binary matrix that recorded the presence of bands as a plus (+) and absence of bands as minus (-). Semi-automated scoring was performed with SAGA^{MX}software (Version 4, LI-COR, Lincoln, NE, USA). Scores were also

manually edited to make corrections to the automated score where appropriate.

Data analysis

Polymorphic bands were scored as plus (+) and minus (-) and converted to 1 or 0 when compiled into a data matrix. The data matrix was used to perform cluster analysis on the basis of the average linkage method, known as the Unweighted Pair Group Method (UPGMA) using PAUP software (Swofford, 1998), while the "goodness of fit" of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between dissimilarity and the cophenetic matrix derived from the dendrogram (Sneath & Sokal, 1973). Polymorphism information content (PIC) provides information on the informativeness of a marker or an estimate of the discriminatory power of the locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values in this study were calculated using the algorithm: PIC (or the diversity index of Nei, 1973)

$$= 1-\sum_{i=1}^{n} fi^{2}$$

where f is the frequency of i^{th} allele averaged across loci. The marker index (MI) was calculated for the AFLP markers by applying the formulae given by Powell *et al.* (1996) and Smith *et al.* (1997). MI = % polymorphism x PIC and % polymorphism = 100 x number of polymorphic bands/ total number of bands in that assay unit.

Calculation of coefficient of coancentry (f)

The estimates of coefficient of coancentry (f) were taken from Messmer *et al.* (1992), wherein f was calculated as described by Falconer & Mackay (1996). For all pair of lines without known parentage, f was set to zero. Two lines were designated as unrelated if their coancentry was smaller than 0.1. Accordingly, for two inbreds related by pedigree with coancentry f and unknown marker genotypes of their ancestors an estimates of their GDs can be obtained as: $G\hat{D} = \overline{GD}(1\text{-}f)$, and if the GD of two individuals is known their coancentry can be estimated by:$\hat{f}$ = 1-GD/$\overline{GD}$ where $\overline{GD}$ refers to the mean genetic distance of unrelated homozygous lines from the respective germplasm group with regard to the investigated set of marker loci and GD is the genetic distance between the two individuals.

Estimation of genetic distances

Estimates of GD among the 21 inbred lines combinations within each set were computed for the AFLP marker system using the formula given by Nei & Li (1979) which is $GD_{ij} = (N_i+N_j-2N_{ij})/(N_i+N_j)$. Here GD_{ij} is the genetic distance between two inbred lines i and j. N_{ij} is the number of common bands in line i and j, and N_i and N_j are the total number of bands in line i and j, respectively, with regard to all primer pair combinations of AFLP. Thus, GD reflects the proportion of bands in common between the two inbred lines and it may range from 0 (identical profile for two inbreds) to 1 (no shared bands).

The Dendrogram

UPGMA was used to produce the dendrogram on the basis of AFLP markers and 21 inbreds (Figure 1), rather than Ward since the main aim of this research was to study the overall pattern of genetic diversity and not to maximize distance between the morphological traits as in case of populations. Warburton *et al.* (2002) used UPGMA to analyze CIMMYT lines for the same reason.

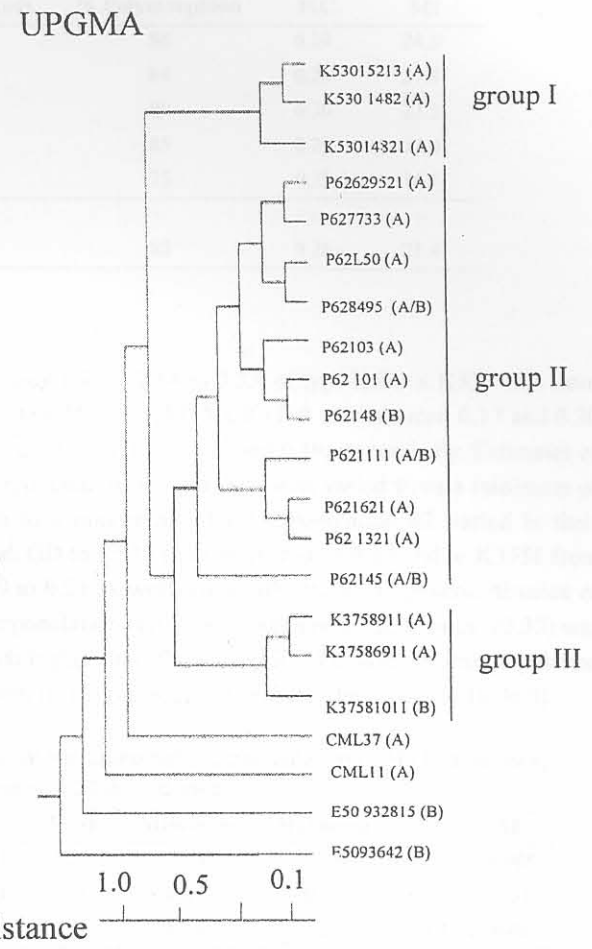

Figure 1 The dendrogram obtained after analysis of the genetic diversity of twenty-one Tanzanian inbred using the Unweighted Pair Group Method.

Results

Degree of polymorphism

The degree of polymorphism was measured by the number of polymorphic bands, percentage polymorphism, polymorphic index content, and marker index as shown in Table 2. The average marker index and the average PIC values for the five primer combinations collectively were 21.4 and 0.26, respectively. Results showed that the largest marker index (24.9) was revealed by the primer pair combination *EcoR*1-ACA+*Mse*1-CCC while the assay unit *EcoR*1-ACA+*Mse*1-CGC showed the lowest index (16.5). The marker indices for the remaining assay units were in between. The total number of fragments (monomorphic and polymorphic bands) per primer pair combination varied from a minimum of 38

(primer pair combination *EcoR*1-ACA+*Mse*1-CGC) to a maximum of 67 fragments (primer pair combination *EcoR*1-ACA+*Mse*1-CCC). The total number of all AFLP markers in this study was 250 fragments, of which 208 fragments were polymorphic with the fragment sizes ranged from 50 base pairs to 456 base pairs (primer combinations not shown). Further more, results showed that the highest PIC (0.30) value was showed by the primer pair combination *EcoR*1-ACA+*Mse*1-CAG and primer pair combination *EcoR*1-ACA+*Mse*1-CGC revealed the lowest PIC (0.22) value. The PIC values for the remaining primer pair combinations were in between.

Table 2 Degree of polymorphism and information content for five primer combinations applied to 21 Tanzanian inbred lines.

*EcoR*1	*Mse*1	Total fragments	Polymorphic bands	% Polymorphism	PIC	MI
1. ACA	CCC	67	58	86	0.29	24.9
2. ACA	CTG	40	34	84	0.26	21.8
3. ACA	CAG	50	40	80	0.30	23.2
4. ACA	CCG	55	47	85	0.24	20.4
5. ACA	CGC	38	29	75	0.22	16.5
Total		250	208	-	-	-
Average		50	41.6	82	0.26	21.4

% Polymorphism = (Polymorphic bands x 100)/ Total fragments

MI = Marker index = % Polymorphism x PIC

Genetic distance between inbred combinations of related and unrelated lines

Comparison of GD estimates between inbreds from the same and different heterotic groups were restricted to pairs of unrelated (f = 0) lines to minimize confounding effects due to relatedness (Table 3). Genetic distance estimates for the line combinations of 21 inbred lines ranged from a minimum of 0.14 (intrapopulation) to a maximum of 0.45 (interpopulation) with a standard deviation for individual estimates varying from 0.02 to 0.06, respectively. Genetic distance estimates for unrelated line combinations within population 62 ranged from 0.14 to 0.33; of type K530 x K530 was from 0.15 to 0.18 and K3758 x K3758 was between 0.17 and 0.20 with a mean of 0.23, 0.16 and 0.19, respectively. Estimates of interpopulation line combinations varied from a minimum of 0.16 to a maximum of 0.45. Population 62 varied in their mean GD to K530 lines from 0.16 to 0.23 and to K3758 from 0.19 to 0.23 as well. Generally, the mean genetic distance of interpopulation (crosses between heterotic groups) (0.32) was much higher than the mean GD of crosses in intrapopulation groups (0.19) (crosses within heterotic groups) (Table 3).

Table 3 Summary of mean, maximum and genetic diversity of individual line combinations calculated from AFLP data using five primer combinations for various groups of related and unrelated pairs of 21 inbred lines.

Type of population	Crosses	#	Mean	Minimum	Maximum	SD	SE
A. Intrapopulations	1. Population 62 x Population 62	44	0.23	0.14	0.33	0.05	0.008
	2. K530 x K530	3	0.16	0.15	0.18	0.02	0.01
	3. K3758 x K3758	3	0.19	0.17	0.20	0.02	0.01
Mean (Intrapopulation)			0.19	0.15	0.23		
B. Interpopulations	1. Population 62 x K530	30	0.23	0.17	0.30	0.04	0.007
	2. K530 x K3758	9	0.24	0.16	0.27	0.04	0.01
	3. Population 62 x CML lines	20	0.31	0.25	0.36	0.03	0.006
	4. K530 x CML lines	6	0.25	0.16	0.32	0.06	0.02
	5. Population 62 x K3758	33	0.28	0.16	0.40	0.06	0.01
	6. Population 62 x Ecuador	22	0.32	0.21	0.38	0.05	0.01
	7. K530 x Ecuador	6	0.35	0.31	0.38	0.02	0.03
	8. K3758 crosses	12	0.34	0.25	0.42	0.06	0.01
	9. CML crosses	6	0.39	0.31	0.43	0.06	0.02
	10. Ecuador crosses	6	0.45	0.45	0.45	-	-
Mean (Interpopulation)			0.32	0.24	0.37		
Total		200					

= refers to the total number of individual crosses in that particular cross

Correlation coefficients between GD and coancentry coefficient (f)

The coancentry coefficient (f) and GD estimates represent fundamentally different concepts for measuring genetic diversity of related individuals (Table 4). Results showed that the coefficient of coancentry (f) for the flint type inbreds was 0.14, while the dent type lines exhibited a f value of 0.20.

Furthermore, correlation coefficients between GD and the *f* values revealed that the flint type inbreds had a correlation value of –0.96. While the dent type inbreds produced a correlation coefficient between GD and coefficient of coancentry of -0.94.

Table 4 Coefficient of coancentry and correlation coefficients (r) between genetic distances and coancentry coefficients (*f*) of maize lines.

Maize germplasm	Number	*f*†	r(GD, *f*)	Marker used
Pop 62 (Flint)	5PC	0.14	-0.96	AFLP
K3758 (Dent)	5PC	0.20	-0.94	AFLP

† = Mean of (*f*) values for all pairs considered
PC = Number of primer pair combinations used

UPGMA dendrogram

The dendrogram or the genetic similarity/dissimilarity tree produced in this study is presented in Figure 1. In this dendrogram, there are three main distinct groups of inbred similarities, namely groups 1 to 3. The lines in group I are: K53015213, K5301482 and K53014821. Group II, which is the largest group was comprised of lines developed from population 62. These lines were: P629521, P627733, P62L50, P628495, P62103, P62101, P62148, P621111, P621621, P621321 and lastly P62145. There were four subgroups of lines within group II. These subgroups were: Subgroup 1 (lines P629521, P627733, P62L50 and P628495); Subgroup 2 (lines P62103, P62101 and P62148); and Subgroup 3 (lines P621111, P621621 and P621321). Then lastly, P62145 was an outlier in this main group. The third main group comprised of lines that originated from a recombinant line K337 x K358, were: K3758911, K37586911 and K37581011. Finally, the dendrogram revealed that the CIMMYT lines (CML37 and CML11) and the Ecuador lines (E50932815 and E5093642) were also outliers.

Discussion

In general, each of the five AFLP primer combinations used in this study discriminated efficiently between most of the unrelated lines. The average marker index in this material was 21.4 (Table 2) which was slightly higher than that reported by Lübberstedt *et al.* (2000) in European maize. Their marker index was 16.4. The larger marker index in this study was possibly attributable to the high average percent of polymorphic bands per lane, while the PIC values were about equal or slightly lower than those of Lübberstedt *et al.* (2000). Similar results were reported for US maize germplasm (Pejic *et al.*, 1998), soybean (Powell *et al.*, 1996) and wheat (Bohn *et al.*, 1999). The higher marker index and PIC value of the AFLP markers in comparison with other marker systems like SSR, RFLPs, RAPDs, together with high reproducibility proves that AFLP markers are a valuable tool for identification of maize inbreds, plant variety protection and registration as well as patenting germplasm (Melchinger *et al.*, 1998a, b; Lübberstedt *et al.*, 2000). AFLP have added advantages in terms of reliability, reproducibility, discrimination, standardization and cost effectiveness. Furthermore, the results showed that primer pair *EcoR*1-ACA+*Mse*1-CAG was the most discriminative or informative because it revealed the highest PIC value (0.30) and primer pair *EcoR*1-ACA+*Mse*1-CGC was the least discriminative because it had the lowest PIC value (0.22).

One of the aims of this study was to test and evaluate inbred lines as potential parents for developing commercial hybrids. It was demonstrated in the study that the GDs of genotypes from different germplasm groups (intergroup crosses) have on average significantly greater GDs than combinations of genotypes from the same germplasm group (intragroup crosses) (Table 3). This observation is consistent with the genetic theory, and those of Melchinger *et al.* (1998a, b) and Pejic *et al.* (1998) which states that intergroup crosses exhibit higher genetic distances between lines and lower genetic distances between lines in the intrapopulation crosses. Breeders have used the coancentry [devised by Malecot's (1948)] as an indirect way of measuring genetic similarities between related individuals. It was observed that r (GD, *f*) values (Table 4) in both flint and dent kernel lines were much higher than in maize in general (Messmer *et al.*, 1993; Smith *et al.*, 1997) and other reported crops (Graner *et al.*, 1994; Schut *et al.*, 1997). The negative sign of the r (GD, *f*) means that in this case as one trait increases, the second tends to decrease. The main reasons for the higher r (GD, *f*) in this study could be due to fundamental differences between the two marker systems used. The AFLP marker system was used, which scores bands on a biallellic basis, while Messmer *et al.* (1993) and Smith *et al.* (1997) used RFLP, which usually scores multiple bands (alleles) per locus (Lübberstedt *et al.*, 2000). Secondly, the AFLPs are capable of assaying more genetic loci than RFLP or SSR (Myburg *et al.*, 2001; Botha *et al.*, 2004; Liu & Cordes, 2004). Thirdly, the higher r (GD, *f*) could be due to the fact that AFLPs are able to amplify potentially more conserved organellar DNA sequences than the RFLP (Vuylsteke *et al.*, 1999). Melchinger (1993) reported that high r (GD, *f*) estimates in maize suggest that if the pedigree data are correct, these correlations (GD, *f*) do provide reliable descriptors of genetic similarity in maize

The dendrogram (Figure 1) revealed that the three recombinant inbreds, developed from K530 germplasm, clustered together and showed some level of genetic similarity with group II, which was comprised of inbreds originating from population 62. This implies that there are some genetic similarities between the recombinant inbreds derived from the K530 germplasm and those developed from population 62. This could be due to gene introgressions between the two sources of germplasm or between the inbreds *per se* or both. The intermingling of genes between the two sources could be via deliberate breeding or through contamination or both. These observations are vital to plant breeders in the sense that they should know the background history and the genetic purity of sources of germplasm they are working with. This, in turn, will help the breeders to develop inbred lines without linkage drag that happens due to use of contaminated germ-

plasm.

In general, the AFLP method could clearly distinguish all the inbreds and clustered them into appropriate groups according to the established pedigrees. However, there were a few discrepancies, especially in the placement of individual inbreds according to the source of germplasm from which they have been extracted. For example the grouping of all recombinant inbreds that were developed from K337 x K358 germplasm clustered with the group containing inbreds extracted from population 62. It was also observed that there were a lot of subgroups of inbreds derived from population 62, implying that this population is genetically diverse. Furthermore, line P62145, although clustered in group II, did not cluster with any of the subgroups. Monforte *et al.* (2003) reported similar discrepancies of clustering with melon inbreds, when using SSR data and comparing population of origin and heterotic grouping, and also a result consistent with high level of genetic diversity within source populations of inbreds. The CIMMYT maize lines, which have different sources of germplasm, were grouped in several groupings as expected. The Ecuador lines, however, according to their pedigree were supposed to group together but fell into different groups. This could be due to contamination or small mutations occurring in one of these lines.

Conclusions

AFLP markers for genetic diversity studies in maize have been employed to investigate the relationship between GD and hybrid performance/heterosis for yield and MPH (Melchinger *et al.*, 1998a, b; Lübberstedt *et al.*, 2000). In this study, however, it was observed that the AFLP markers have another added advantage of producing the best results for measuring correlations between coancentry coefficient and genetic distance of maize inbreds. It had the highest f values as compared to similar studies reported by Smith *et al.* (1997) and Messmer *et al.* (1993) etc. Furthermore, this study revealed that each line was uniquely identified when the five AFLP primer combinations were used and the grouping of the lines was almost consistent with their established pedigree (Figure 1). This research also showed that inbred lines derived from intrapopulations as well as recombinants can have lower genetic distances than inbreds derived from open pollinated varieties (Tables 3), and might suffer from the deleterious effect of inbreeding depression and degeneration. Finally, it is important to note that inbreds which are similar can only be crossed among themselves if (i) they can show an increased level of heterosis for the target trait; (ii) these inbreds could complement one another for those genes which are missing in each other and these genes are able to improve the agronomical traits of the resulting hybrids; and (iii) are needed to produce large quantities of seed which single crosses are not able to produce.

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