# Identification of Grey Leaf Spot Resistance in F2 Maize (Zea Mays L.) Populations using Simple Sequence Repeat Markers (SSR)

**Lagat Nicholas[1]*, Maurice E Oyoo [2], Murerwa Pactrick[2] and Owuoche James[2]**

[1]University of Eldoret, Biotechnology Department Box 1125 Eldoret, Kenya

[2]Egerton University, Department of Crops, Horticulture and Soil Science, P.O Box 536- 20115 Egerton, Kenya

**Corresponding Author:** Lagat Nicholas; Email: nicholaslagat3@gmail.com

## Abstract

Maize (*Zea mays* L.) is an important cereal crop worldwide especially in Sub-Saharan Africa. The yields of maize are, however, relatively low in Kenya largely due to foliar disease that significantly led to inconsistencies in productivity of maize genotypes in various environments. Identification and utilization of disease resistance mechanism and development of resistant cultivars is crucial in disease management. The objective of the study was to identify SSR markers linked to GLS resistance genes among the selected $F_2$ population. A total of 23 genotypes were selected based on their disease severity in the field. Initially these genotypes were selected from a group of 20 $F_2$, 9 parents and two checks resistant (R) and susceptible (S) to GLS. Symptomatic fresh leaves were used in the extraction of genomic deoxyribonucleic acid (DNA) and amplification done using markers linked to grey leaf spot disease. A total of four SSR markers were screened for polymorphism among the resistant $F_2$ population. Among, the four SSR markers (Cpr1-117757, Bnlg 1258**,** Bnlg 1194, and Phi 031) only one primer (Cpr1-117757) was found to be polymorphic and they were used for finding out resistant gene harbouring plants. The banding pattern of all screened genotypes were scored as either present (+) or absent (-) of the targeted resistant gene. Thirteen $F_2$ population (CKDHL 120312/CKL 05017, CKL 15537/CML 568, CKDHL 120312/$S_5$96-15-1-1, KTLN 10123/$S_5$96-15-1-1, CKL 05022/$S_5$96-15-1-1, CKDHL 153502/$S_5$96-15-1-1, CKDHL 120312/CML 395, CKL 05022/ CML 395, CKDHL 153502/CML 395, CKL 15537/CML 395, CKL 05022/CKL 05017, CKL 15537/CKL 05017 and CKDHL 153502/CML 568), eight parents (CKDHL 12312, KTLN 10123, CKL 05022, CKDHL 153502, CKL 05017, CML 568, $S_5$96-15-1-1 and CML 395) and two commercial checks (H512 and H614) which were categorized as the resistance and moderately resistance genotypes under artificial inoculation revealed different banding patterns. The information provided by the identified marker would be very useful in breeding programs to select maize genotypes harbouring GLS resistance genes.

**Keywords:** Germplasm, GLS, Polymorphism, Segregating and SSR

### 1.0 Introduction

In rural and urban households in Kenya, maize has become a significant crop in many families because of its potential as a source of dietary fibre, protein and phytochemical compounds and hence contribution to nutritional security (Galani *et al.*, 2022). The inheritance of grey leaf spot is a complex trait controlled by multiple genes with additive effect (Kuki *et al*., 2018). It is challenging to improve complex traits which are influenced by environment through conventional breeding, but the use of molecular markers enhances and fasten the selection process although it

requires efficient field screening so as to relate phenotypic characteristics with DNA variations. Quantitative trait loci (QTL) for GLS resistance have emerged as effective method of identifying candidate genes governing the traits to be selected in crops (Sun *et al*., 2021a). The identification of SSR markers linked to disease resistant is crucial, therefore, for understanding the molecular basis of these traits quite important in future breeding.

Marker assisted selection (MAS) for selection of genes linked to a specified trait with measurable differences is an important technique in molecular breeding. The technique is used for measuring effect of complex traits linked to a specific genomic region (Maloy & Hughes, 2013). In comparison to GWAS and QTLs studies, MAS have the advantage of utilizing few numbers of lines that need to be tested. Since many lines can be discarded after MAS at an early generation, this permits a more effective breeding design (Hasan *et al*., 2021). Effectiveness of marker assisted selection depends on molecular marker to be used, thus significant progress in crop disease resistance breeding has been achieved by MAS. However, the study of GLS resistance on maize using SSR markers has not been widely documented.

Different methods have been used for screening for GLS resistance under laboratory, greenhouse and field environments. Laboratory is the most important method because it saves time and cost. Marker-assisted selections, molecular analysis, linkage Mapping, and selective phenotyping can be utilized in modern laboratories (Kuki *et al.,* 2018b; Kibe *et al.,* 2020a). Using molecular marker systems, genomic regions responsible for GLS resistance with specified marker can be located. molecular breeding techniques allows rapid identification of markers, for trait mapping in plant breeding. This is a sole proofs on the molecular analysis available in crops (Nyanapah *et al.,* 2022). Genetic mapping and genome-wide association has been done using single- nucleotide polymorphism (SNP) markers (Sun *et al*., 2021b). Combining molecular analysis with field screening can be the most effective and reliable breeding method than the use of field screening alone in identification of important traits in crop improvement.

Studies of molecular analysis using SSR markers related to grey leaf spot resistance in maize are limited. According to Omondi, (2022) and Du *et al*, (2020c), quantitative trait loci (QTLs) closely linked with *qGLS_YZ2-1* region and GLS resistance using SSR markers has been done. However, molecular analysis using marker assisted selection (MAS) in GLS breeding in maize improvement in Kenya and markers specific to GLS are scarcely researched up to date.

## 2.0 Materials and methods

### 2.1 Plant materials

A total of 23 (table 1) maize germplasm, from previous screening and selection across three environments representing parents, $F_2$ populations and the commercial checks were selected for molecular analysis. Each of the genotypes was grown at the University of Eldoret, glass house in three replicates (0°37'N and 35°15'E, approximately 2,143 m. a. s. l.). The mean maximum and minimum temperatures were 26 and 10 $^0$C.

**Table 1: Maize germplasm used in the study**

| CODE | GENOTYPES | CLASSIFICATION |
|---|---|---|
| G1 | CKDHL 120312/CKL 05017 | R |
| G10 | CKL 15537/CML 568 | MR |
| G11 | CKDHL 120312/S5 96-15-1-1 | R |
| G12 | KTLN 10123/S5 96-15-1-1 | R |
| G13 | CKL 05022/S5 96-15-1-1 | R |
| G14 | CKDHL 153502/S596-15-1-1 | R |
| G15 | CKL 15537/S5 96-15-1-1 | MS |
| G16 | CKDHL 120312/CML 395 | R |
| G17 | KTLN 10123/CML 395 | MR |
| G18 | CKL 05022/CML 395 | R |
| G19 | CKDHL 153502/CML 395 | R |
| G2 | KTLN 10123/CKL 05017 | MR |
| G20 | CKL 15537/CML 395 | MR |
| G21 | H512 | R |
| G22 | H614 | MR |
| G23 | CKDHL 120312 | MR |
| G24 | KTLN 10123 | R |
| G25 | CKL 05022 | MR |
| G26 | CKDHL 153502 | MR |
| G27 | CKL 15537 | MR |
| G28 | CKL 05017 | MR |
| G29 | CML 568 | MR |
| G3 | CKL 05022/CKL 05017 | MR |

## 2.2 Deoxyribonucleic acid (DNA) extraction

Total genomic DNA was extracted from 60 days old maize leaves expressing symptoms of grey leaf spot (GLS) harvested from greenhouse plants. The harvested leaves were labelled and immediately put in icebox and taken to the laboratory where they were placed in -80°C for ease of grinding. Prepared Cetyltrimethylammonium bromide (CTAB) and modified protocol as suggested by (Olufemi *et al*. 2008) was used to extract the DNA where 100 mg leaf samples were weighed and crushed to form a homogenous paste in 500 µl CTAB buffer (10% SDS, 0.5 M EDTA, 1 M Tris-HCl with a final pH of 8.0). 10 ul of 100mg/ml RNaseA to remove RNASES was added and the homogenate transferred into 1.5ml Eppendorf tube and incubated at 65 °C water-bath for 30 minutes. The tube was inverted 5-6 times after every five minutes during the extracted sample was then cooled down in fridge for 15 minutes and 200ul of 6M Ammonium Acetate added and vortexed. The mixture was kept in fridge at 4°C for 15 minutes. Using Eppendorf centrifuge, the mixture was centrifuged for 5 minutes at 13000rpm. The supernatant was transferred to a new Eppendorf tube and 50 µl of 10% CTAB preceding addition of 700 ul of chloroform: isoamyl alcohol (24:1) and gently mixed by inversion. The mixture was centrifuged at 13,000g for 5 minutes. The upper phase (approx. 500ul) was transferred to a new Eppendorf tube and DNA precipitated by addition of 350ul ice cold isopropanol, the tubes were gently inverted and kept at -20°C for 15 minutes. The precipitated DNA was pelleted by centrifuging at 14000 rpm for 20 minutes followed by 70% and 90% ethanol washing the pellets. The pellets were air dried on a clean bench and dissolved in 100ul 1x TE (tris EDTA) buffer.

## 2.3 DNA Quantification

The quality of DNA was determined by running it on 1% agarose gel with 1x TBE buffer (Trizma base with EDTA and boric acid; pH was adjusted to 8.0 with NaOH) at 100 V for 45 minutes. As a check for DNA quality and quantity, all samples from infected genotypes were successfully subjected to quantification using Nanodrop spectrophotometer (ND-2000, UV-Vis spectrophotometer) (Table 2)

Table 2: DNA Concentration of 23 genotypes

| Nucleic Acid | Unit | A280 (Abs) | 260/280 | Sample type | Factor | # |
|---|---|---|---|---|---|---|
| 89.9 | $ng/\mu l$ | 3.637 | 0.49 | DNA | 50.00 | 1 |
| 77.3 | $ng/\mu l$ | 1.013 | 1.53 | DNA | 50.00 | 2 |
| 75.6 | $ng/\mu l$ | 1.235 | 1.22 | DNA | 50.00 | 3 |
| 338.9 | $ng/\mu l$ | 3.595 | 1.89 | DNA | 50.00 | 4 |
| 48.6 | $ng/\mu l$ | 0.744 | 1.31 | DNA | 50.00 | 5 |
| 12.4 | $ng/\mu l$ | 0.237 | 1.04 | DNA | 50.00 | 6 |
| 13.9 | $ng/\mu l$ | 0.255 | 1.09 | DNA | 50.00 | 7 |
| 74.3 | $ng/\mu l$ | 0.972 | 1.53 | DNA | 50.00 | 8 |
| 79.0 | $ng/\mu l$ | 1.041 | 1.52 | DNA | 50.00 | 9 |
| 65.7 | $ng/\mu l$ | 0.954 | 1.38 | DNA | 50.00 | 10 |
| 113.4 | $ng/\mu l$ | 1.395 | 1.63 | DNA | 50.00 | 11 |
| 190.1 | $ng/\mu l$ | 2.372 | 1.60 | DNA | 50.00 | 12 |
| 189.9 | $ng/\mu l$ | 2.374 | 1.60 | DNA | 50.00 | 13 |
| 178.3 | $ng/\mu l$ | 1.849 | 1.93 | DNA | 50.00 | 14 |
| 170.0 | $ng/\mu l$ | 2.497 | 1.36 | DNA | 50.00 | 15 |
| 183.2 | $ng/\mu l$ | 2.063 | 1.78 | DNA | 50.00 | 16 |
| 99.6 | $ng/\mu l$ | 1.124 | 1.77 | DNA | 50.00 | 17 |
| 142.8 | $ng/\mu l$ | 1.530 | 1.87 | DNA | 50.00 | 18 |
| 1370.8 | $ng/\mu l$ | 12.893 | 2.13 | DNA | 50.00 | 19 |
| 407.6 | $ng/\mu l$ | 4.215 | 1.93 | DNA | 50.00 | 20 |
| 36.1 | $ng/\mu l$ | 0.470 | 1.54 | DNA | 50.00 | 21 |
| 16.6 | $ng/\mu l$ | 0.258 | 1.28 | DNA | 50.00 | 22 |
| 201.2 | $ng/\mu l$ | 2.220 | 1.88 | DNA | 50.00 | 23 |

## 2.4 Polymerase chain reaction (PCR) amplification

The extracted DNA was amplified using Eppendorf master cycler gradient PCR. Four SSR primers pairs were selected based on their linkage with grey leaf spot resistance gene. The primer codes and base pairs are shown in table 3.

**Table 3: Summary of SSR primers used in the study**

| SN | Primer | Sequence | Ta $^0$C |
|---|---|---|---|
| 1 | Bnlg 1258 | F: GGTGAGATCGTCAGGGAAAA | 49(53) |
| | | R: GAGAAGGAACCTGATGCTGC | |
| | | TGAACTACGCGCTCAATGGTCCACGAAACAAGTACGA | |
| 2 | Cpr1-117757 | TTCGACACTCGAACTTCAAGCTCCCCTCAGACCCAAGC | 52 (48) |
| 3 | Bnlg 1194 | F: GCGTTATTAAGGCAAGCTGC | 58(52) |
| | | R: ACGTGAAGCAGAGGATCCAT | |
| 4 | Phi 031 | F: GCAAGAGGTTACATGAGCTCACGA | 45(57) |
| | | R: CCAGCGTGCTGTTCCAGTAGTT | |

**Ta, annealing temperature, Tm, Melting (temperature)**

## 2.4 Visualization of the PCR bands

A 1% Agarose gel was prepared by mixing 1.0 g of Agarose with 100 ml 1x TBE (Tris- HCL Boric Acid EDTA) buffer. The solution was then heated in a microwave for proper mixing. The gel was left to cool to approximately 60°C then 3 µl of nucleic acid gel stain Ethidium Bromide was added. The gel was then cast in trays and combs carefully placed and solidification allowed occurring. The PCR products obtained were then mixed with 1 µl of the loading dye (orange G) and 5µl of the sample loaded in each well. The data on band separation was scored as present (+) or absent (-) for the genes responsible for GLS diseases in maize.

## 3.0 Results

### Detection of the gene linked to GLS disease in maize

Molecular analysis of the resistant gene by SSR markers revealed the present of GLS genes in the resistant genotypes (Table 3). The PCR product of for these genotypes were digested into different fragments with the CPrI-117757, while it remained uncut when treated with BnIg 1258, Bnlg 1194 and Phi 031, suggesting that the GLS gene is located in 164bp and is in homozygous state (Fig. 1), but no amplification was obtained from susceptible genotypes.

Six genotypes possessed the susceptible genes. The gene were grouped into three different clusters due to comparison in disease severity in the field. CKL 05022/S596-15-1-1 and CKDHL 120312/CML 395 were grouped in cluster 1, with high level of resistant, CKDHL 120312/CKL05017, CKDHL 120312/S596-15-1-1, KTLN 10123/S596-15-1-1, CKDHL 153502/S596-15-1-1, CKL 05022/CML 395, CKDHL 153502/CML395 and KTLN 10123 from cluster 2 had severity level of 2, CKL 15537/CML 568, CKL 15537/CML395, CKDHL 120312, CKL 05022, CKDHL 153502, CML 568 and CKL 05022/CKL05017 severity of 3. The susceptible were CKL 15537/S596-15-1-1, KTLN 10123/CML 395, KTLN 10123/CKL05017, CKL 05017 and CKL15537.

**Table 4: Presence (+) or absence (-) of resistant genes linked to GLS in 23 screened genotypes**

| No | Genotypes | GLS Primers | | | | Field data |
|---|---|---|---|---|---|---|
| | | CPrI-117757 | Bngl 1258 | Bnlg 1194 | Phi 031 | GLS |
| 1 | CKDHL 120312/CKL05017 | + | - | - | - | 2.65 |
| 2 | CKL 15537/CML 568 | + | - | - | - | 3.7 |
| 3 | CKDHL 120312/S596-15-1-1 | + | - | - | - | 2.55 |
| 4 | KTLN 10123/S596-15-1-1 | + | - | - | - | 2.25 |
| 5 | CKL 05022/S596-15-1-1 | + | - | - | - | 1.85 |
| 6 | CKDHL 153502/S596-15-1-1 | + | - | - | - | 2.72 |
| 7 | CKL 15537/S596-15-1-1 | - | - | - | - | 5.77 |
| 8 | H614 | - | - | - | - | 5.28 |
| 9 | CKDHL 120312/CML 395 | + | - | - | - | 1.83 |
| 10 | KTLN 10123/CML 395 | - | - | - | - | 4.42 |
| 11 | CKL 05022/CML 395 | + | - | - | - | 2.52 |
| 12 | CKDHL 153502/CML395 | + | - | - | - | 2.38 |
| 13 | KTLN 10123/CKL05017 | - | - | - | - | 4.53 |
| 14 | CKL 15537/CML395 | + | - | - | - | 3.65 |
| 15 | H512 | + | - | - | - | 2.78 |
| 16 | CKL 05017 | - | - | - | - | 4.25 |
| 17 | CKDHL 120312 | + | - | - | - | 3.22 |
| 18 | KTLN 10123 | + | - | - | - | 2.65 |
| 19 | CKL 05022 | + | - | - | - | 3.22 |
| 20 | CKDHL 153502 | + | - | - | - | 3.83 |
| 21 | CKL15537 | - | - | - | - | 4.35 |
| 22 | CML 568 | + | - | - | - | 3.37 |
| 23 | CKL 05022/CKL05017 | + | - | - | - | 3.77 |

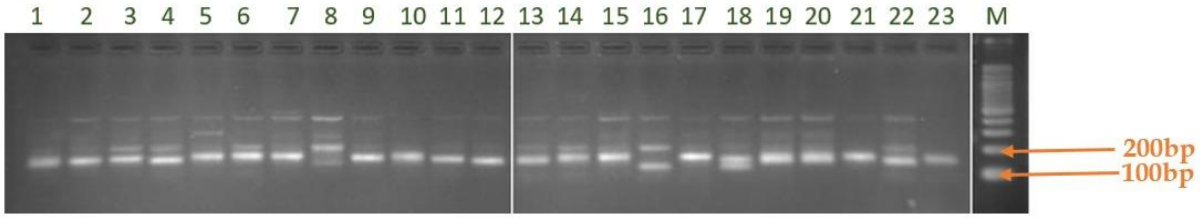


**Figure 1: SSR marker for identification of GLS resistant gene among maize genotypes alongside two checks, resistant (H512) and susceptible (H614) commercial hybrids.**

Based on SSR analysis, the specific resistant gene for grey leaf spot in maize among the 23 genotypes was located at 164bp (Fig 1). Therefore, six genotypes did not express the presence of resistant gene with the remaining three primers; hence it was classified as absent (-) of the resistant gene (Table 4). The three primers for GLS (Bngl 1258, Bnlg 1194 and Phi 031) did not detect any genes for grey leaf spot disease across all the genotypes and their respective parental lines. However, the CPrI 117757 primer detected the presence of genes linked to grey leaf spot in almost

all the genotypes selected except genotypes CKL 15537/S596-15-1-1, KTLN 10123/CML 395, KTLN 10123/CKL 05017, CKL 05017 and CKL 15537. The banding pattern for all the 20 genotypes showed the presence of resistance GLS genes as well as the parental resistant check (H512).

## 4.0 Discussion

Knowledge about molecular breeding and technology in crops especially breeding disease resistant genotypes is critical for genotypes utilization, and variety development. The primary goal of any variety development and crop improvement program is to target grain yield and yield components, but breeding for disease resistant, fodder quantity and quality and other traits of economic is equally important. To achieve this balance, any breeding program needs to have gene pool where sufficient genotypes recombination is being created for future references. Creating this gene pool requires mixed genotypes that have potential characteristic of fulfilling farmer's interest. Combination of both molecular analysis and field morphological data have been reported extensively in maize breeding (Gedil & Menkir, 2019), similarly, the current study give insights of the importance of the two analyses.

The results obtained from this study show resistant genotypes among evaluated $F_1$ generation and their parents in GLS resistance. These results suggest that some of the genotypes have the resistant gene located at 164bp which is in concurrence with earlier studies that conventional and quantitative PCR produced a 164 bp fragment in *Cercospora zeae-maydis* isolates (Korsman *et al*., 2012). Also, field data on disease severity corresponded with the molecular data that all resistant genotypes at the field had the resistant gene. Therefore, this information could be used as a base for the development of varieties that have resistance in disease pressured environments. The cross between genotype KTLN 10123 which was rated as the most resistant parent and $S_5$ 96-15-1-1 which was moderately resistant at the field produced among the most resistant combination in the field and confirmed during molecular analysis, this study was similar with the report by Dhami *et al*. (2015), who reported that crosses between resistant and the most susceptible lines resulted in resistant hybrids due to the predominance of additive nature of gene actions and major dominant effects of some genes.

Any successful maize breeders require a stable resistance variety against grey leaf spot. Conventionally, breeders have relied on the resistant hybrids from Kenya seed company, however, with time, these hybrids have been compromised by GLS disease because of environment fluctuation. Therefore, resistant gene located in 164bp become important resistance source to many breeders in Kenya. Currently, quantitative trait loci (QTLs) has been successfully employed in the development of resistance varieties (Benson *et al*., 2015). Furthermore, study on GWAS on GLS resistance maize has been done (Hu *et al*., 2024a ; Nyanapah *et al*., 2022 ; Zhu *et al*., 2021). However, location of the gene responsible for GLS resistance using molecular markers has proven a reliable approach to accelerate the breeding process. Currently, several markers linked to this locus are available for screening maize genotypes (Korsman *et al.*, 2012), however, their efficacy for identifying resistance gene needs to be evaluated more rigorously. Earlier reported studies seeking resistance against GLS have shown that the SSR markers have high specificity than other markers (Qiu *et al.*, 2021). Reliability and high level of polymorphism of the SSR markers in investigated germplasm will reduce the tracking of resistance genes during the selection process and speed up the breeding cycle.

In the current study, among the resistant genes, genotypes CKDHL 120312/CML 395 and CKL 05022/$S_5$ 96-15-1-1 were the leading. and these genotypes were combination of different maize lines. These genotypes can be used as sources of resistance genes especially in combination of high yielding varieties.

## 5.0 Conclusion

The results of this study provide a more thorough and higher-resolution understanding of the molecular analysis of GLS resistance and provide initial support about gene responsible for resistance to GLS in maize. Plant breeding decisions regarding development and deployment of resistance will accelerate the breeding cycle if only there is better understanding of the molecular breeding for disease resistance.

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