***Review Article***

**Identification of gray leaf spot resistance among selected F2 maize (Zea mays L.) populations using simple sequence repeat markers (SSR)**

**Abstract**

Accelerating maize breeding programs requires a deep understanding of molecular breeding, to select genotypes with desired traits, especially resistance to gray leaf spots. This study used SSR markers to identify GLS resistance genes in F2 maize populations. A total of 23 maize genotypes were evaluated, including 13 F2 populations, 8 parental inbred lines, and 2 commercial checks. DNA was extracted from leaf samples using the CTAB method, purified with a Nanodrop spectrophotometer, and analyzed using 4 SSR markers. The amplified DNA fragments were visualized on a 2% agarose gel, and the bands were scored for the presence (+) or absence (-) of the targeted GLS resistance gene. DNA from 23 genotypes was analyzed, revealing a resistance-associated region at 164 bp. Different banding patterns indicated variations in the GLS resistance gene among genotypes. The study showed a correlation between phenotypic and molecular data, supporting the use of SSR markers for breeding. The total of effective alleles was 5.554 and the number of alleles per primer ranged from 0.00 to 1.00. The observed number of alleles was 1 while the number per primer ranged from 1 to 0 with a mean of 1.48. Based on these, the effective number of alleles was lower than the observed number. Furthermore, variation between groups (SS = 15.25, MS = 5.083) is substantially greater than the variation within groups (SS = 8.90, MS = 0.468), suggesting that most genetic diversity is structured among groups rather than within them. These findings advance molecular breeding strategies for developing GLS-resistant maize varieties, aiding in improving productivity and sustainability in GLS-affected GLS regions.

**Keywords: DNA, SSR, GLS Gene and Allele**

**1 Introduction**

Gray leaf spot (GLS), caused by the pathogens *Cercospora zeae-maydis* and *Cercospora zeina*, is a threat to maize (*Zea may*s L.) cultivation crops globally (Nsibo et al., 2024; Duan et al., 2022). This disease is characterized by necrotic lesions and chlorosis, which can reduce photosynthetic efficiency and lead to yield losses, especially in favourable conditions. The disease produces a large number of spores that can persist in the soil for long periods. The resilience of GLS pathogens enables them to infect hosts for an extended period after release, as shown in studies by Jain et al. (2019) and Dhami et al. (2015). This involves a thorough examination of genetic traits that confer resistance to GLS, aiming to develop maize varieties that are both resilient and high-yielding (Kainyu, 2014). By emphasizing these resistant traits, breeding programs can protect maize crops and promote agricultural sustainability. Prioritizing these efforts can bolster the resilience of maize production and support sustainable agriculture in the face of increasing GLS pressure.

Genetic resistance is a key strategy for managing gray leaf spots in maize, reducing the need for chemical fungicides and promoting sustainable agriculture practices (Hu et al., 2024). However, the complex polygenic nature of GLS resistance poses challenges for genetic analysis and selection. In recent years, molecular marker technologies, such as simple sequence repeat (SSR) markers, have proven valuable for dissecting complex traits and expediting the breeding of disease-resistant crops. SSR markers, known for their high polymorphism, reproducibility, and codominance, are particularly useful for mapping quantitative trait loci (QTL) associated with resistance. The F2 population, resulting from crossing resistant and susceptible maize lines, serves as a valuable resource for studying the genetic basis of GLS resistance. By leveraging SSR markers, this study aims to identify genomic regions linked to resistance, enabling marker-assisted selection (MAS) for GLS-resistant maize lines. The goal is to identify and validate SSR markers associated with GLS resistance in F2 maize populations, offering valuable insights for breeding programs and contributing to the development of durable GLS-resistant maize cultivars.

Simple sequence repeat markers are widely used in plant genetics and breeding due to their high polymorphism, codominance, reproducibility, and ease of use (Zhou et al., 2021; Lehmensiek et al., 2001). They are valuable tools for linkage analysis and QTL mapping, to identify genomic regions associated with GLS resistance. SSR markers can help identify specific resistance alleles for use in marker-assisted selection (MAS) to develop resistant maize varieties more quickly (Zebire, 2020). Molecular marker analysis is essential for studying genetic variability in crops and segregation in F2 populations. This approach utilizes molecular markers to identify genetic variations and effectively track inheritance patterns. Linkage mapping focuses on identifying genetic linkages between traits and markers through statistical analysis providing valuable insights for breeding programs (Bello et al., 2012). By combining phenotypic evaluation of GLS resistance with genotypic data from SSR markers, QTL associated with resistance traits can be identified and validated (Craze et al., 2022; Bekeko et al., 2018). The objective of this study is to identify SSR markers linked to GLS resistance in F2 maize populations, facilitating the development of molecular tools for MAS. This study aims to deepen the understanding of the genetic basis of GLS resistance and provide a practical framework for breeding durable, high-yielding maize cultivars with enhanced disease resilience. This study is expected to significantly contribute to the development of durable, high-yielding maize cultivars with improved disease resilience promoting food security and sustainable farming practices in diverse agricultural systems.

**2 Materials and Methods**

**2.1 Plant materials**

The study evaluated a total of 23 maize genotypes, comprising 13 F**2** populations that were selected based on their resistance to GLS disease during field screenings conducted at KALRO Kakamega and Egerton Field 7. Additionally, the study included 7 parental genotypes and two commercial checks from Kenya Seed Company (Table 1).

**2.2 Total genomic nucleic acid extraction**

Total genomic DNA was extracted from young plant leaf samples using Cetyltrimethylammoniumbromide (CTAB) protocol (Appendix 1) with slight modifications. The slight modifications involved thorough cleaning of DNA during extraction to remove contaminants like proteins, lipids, polysaccharides, and other cellular debris present in the sample. This ensures the highest quality and reliability of the analysis.

**2.3 DNA Quantification**

DNA purity and quantity were determined using Nanodrop spectrophotometry (ND-2000, UV-Vis spectrophotometer). DNA samples were run in a 1% agarose gel containing Ethidium bromide staining dye at a voltage of 150 V and a current of 400 mA for 30 min and visualized on a UV Transilluminator.

**Table 1: List of 23 genotypes of maize along with their check lines used for field screening**

|  |  |  |
| --- | --- | --- |
| **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 × S5 96-15-1-1 | R |
| G15 | CKL 15537 × Ss 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 |

**R-Resistance; MR-Moderately Resistance; MS- Moderately susceptible; CML-CIMMTY lines.**

**2.4 Selection of SSR markers**

A total of 4 sets of SSR markers (Table 2) were selected from earlier publications according to Danson et al. (2008). The SSR markers were selected on the basis that they are linked to the region of the chromosomes that are known to harbour GLS-resistant genes. These were previously reported to have a large PIC content and hence can be used to reveal polymorphism among the evaluated genotypes. Polymerase Chain Reaction (PCR) was done in 96 PCr wells in 23μl final volume containing DNA template (20 ng) 2.0 μl, primer forward (10 μmol/L) 1.5 μl, primer reverse (10 μmol/L) 1.5 μl, 10x PCR buffer 2.0 μl, 10 mM dNTPs (2.5 mM each) 1.8 μl, MgCl2 (1.2 mM) 1.2 μl, Taq DNA polymerase (5 U/μl) 3.0 μl and dd H2O 12.0 μl. The amplification conditions for the PCR profile were initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, specific annealing temperature for each SSR primer for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min and infinite time at 4°C for storage.

**Table 2 List of primer sequences of 4 microsatellite markers (F- forward primer and R - reverse primer).**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S/N** | **Primer** | **Primer Sequence** | **Repeat Motif** | **Ta (°C)** | **Reference** |
| 1 | Bnlg 1258 | F: GGTGAGATCGTCAGGGAAAA  R: GAGAAGGAACCTGATGCTGC | AG (24) | 49 (53) | Danson et al., 2008 |
| 2 | Cpr1-117757 | F: TGAACTACGCGCTCAATGGTCCACGAAACAAGTACGA  R: TTCGACACTCGAACTTCAAGCTCCCCTCAGACCCAAGC | (CAG)4 | 52 (48) | Shiri et al., 2011 |
| 3 | Bnlg 1194 | F: GCGTTATTAAGGCAAGCTGC  R: ACGTGAAGCAGAGGATCCAT | AG (33) | 58 (52) | Krishna et al., 2012 |
| 4 | Phi 031 | F: GCAAGAGGTTACATGAGCTCACGA  R: CCAGCGTGCTGTTCCAGTAGTT | GTAC | 45 (57) | Shiri et al., 2011 |

**2.5 Scoring of the markers**

SSR marker alleles were manually scored from the gel images. A simple numerical scoring method was employed, with 1 denoting the presence of the expected band and 0 denoting its absence.

**2.6 Data analysis**

The markers were quantified in terms of the number of amplicons per primer, percent polymorphism, and polymorphic information content (PIC). Shannon information index was calculated with the following index:

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Where pi is the proportion of each allele (0 or 1)

[The PIC values of individual primers were calculated based on the formula PIC = 2 × F (1- F), where F is the frequency of the bands]. Phylogenetic analysis was carried out to estimate evolutionary relationships among the vanilla accessions. All phylogenetic analyses were done in DARwin 6.0.8 using binary data from the gel image marker scores.

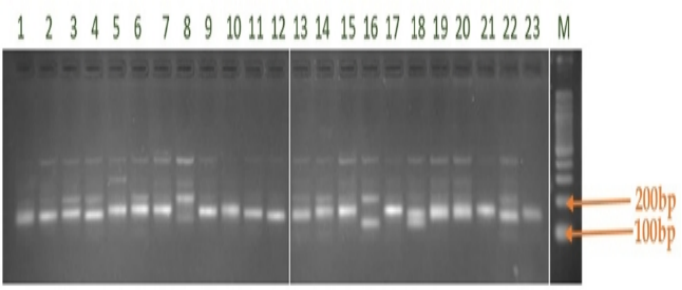
**3 Results**

**3.1 Genetic attributes of SSR markers**

Both the amount and quality of the extracted DNA were suitable for PCR. The range of sample purity was 0.49 to 2.13, and the range of sample DNA concentrations was 12.4 to 1370.8 ng/μl. Every primer yielded the expected product sizes. A single band (scored as homozygous at the amplified loci), no bands (indicating absence of the target loci), or multiple bands (scored as heterozygous at the amplified loci) were observed in some samples Fig.1.

**3.2 Polymorphism of the markers**

Four SSR primers were used in the study, and only one resulted in amplified fragments which varied in size from 164 to 190 bp (Figure 2). The marker was recorded in terms of the observed number of alleles. The number of amplicons per genotype ranged from 1 to 4 with only six genotypes giving one allele each (1,9,10,11,12 and 23).



**Figure 1: Gel electrophoresis results display the amplicons of different genotypes Primer CprI 117757**

**| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |:A represents 500 bp DNA ladder, and Lane 1 is the amplicon for the positive control (CPrI-117757). Lanes 1, 2,3,4,5,6,9,11,12,14,15,17,18,19,20, and 22,23 indicate genotypes that harbour GLS-resistant gene (164bp) and Lanes 7, 8, 10, 13, 16, and 21 indicate isolates that possess non-resistance region (190bp).**

The total of effective alleles was 5.554 and the number of alleles per primer ranged from 0.00 to 1.00. The observed number of alleles was 1 while the number per primer ranged from 1 to 0 with a mean of 1.48. Based on the results, the effective number of alleles was lower than the observed number of alleles (Table .3). The primer with 0 PIC value was monomorphic showing that it could not differentiate between the accessions while the primer with the highest PIC had the highest resolution. For instance, primer Bnlg 1258 and Cprl 117757 had the highest resolution while primer Bnlg 1194 and Phi 034 were monomorphic. Therefore, alleles with high frequencies contribute more to the effective number of alleles in this study.

**Table .3: summarizing the Shannon Information Index for each locus**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Primers** | **Sample size** | **na\*** | **ne\*** | **I\*** | **m** | **PIC** |
| Bnlg 1258 | 23 | 0.7391 | 1.777 | 0.344 | 0.2609 | 0.3855 |
| Cprl 117757 | 23 | 0.7391 | 1.777 | 0.344 | 0.2609 | 0.3855 |
| Bnlg 1194 | 23 | 0.00 | 1 | 0 | 1 | 0 |
| Phi 034 | 23 | 0.00 | 1 | 0 | 1 | 0 |
| **Mean** |  | **0.369** | **1.389** | **0.172** | **0.63** | **0.19** |

\*na = Observed number of alleles, \*ne = Effective number of alleles, \*I = Shannon's Information index, PIC = Polymorphic Information content, m = Major allele frequency

**3.3 Molecular markers, their presence/absence, and how they relate to GLS resistance in maize.**

The molecular banding patterns for the different genotypes, as assessed using GLS primers, are summarized in Table .4, along with their corresponding GLS field data. The results provide a clear indication of the presence (+) or absence (-) of specific molecular markers in the genotypes tested. Notably, the CPrI-117757 primer produced a distinct band (+) in many of the genotypes, suggesting that this primer successfully detected a specific genetic sequence that is present in these genotypes. This banding pattern may be associated with traits relevant to gray leaf spot resistance or other maize characteristics. On the other hand, the primers Bngl 1258, Bnlg 1194, and Phi 031 did not yield any bands in any of the genotypes, indicating that these markers are either non-polymorphic in the context of this study or simply absent in the genotypes under investigation.

**Table .4: Presence (+) or absence (-) of resistant genes linked to GLS**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | GLS Primers | | | | Field data |
| No | Genotypes | 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 |

**3.4 Analysis of Molecular Variance (AMOVA)**

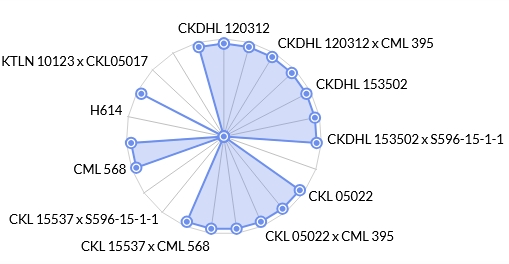
The AMOVA results indicate significant genetic differentiation between groups, as evidenced by the highly significant p-value (0.0001) and a high F-ratio (10.67) (Table 5). The variation between groups (SS = 15.25, MS = 5.083) is substantially greater than the variation within groups (SS = 8.90, MS = 0.468), suggesting that most of the genetic diversity is structured among groups rather than within them. With a total sum of squares of 24.15, the majority of the observed genetic variance is attributed to differences between groups rather than individual variation within groups.

**Table .5: Analysis of Molecular Variance of the 23 genotypes selected.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Source of Variation** | **DF** | **SS** | **MS** | **F-ratio (F)** | **P-value** |
| **Between Groups** | 3 | 15.25 | 5.083 | 10.67 | 0.0001 |
| **Within Groups** | 19 | 8.90 | 0.468 |  |  |
| **Total** | 22 | 24.15 |  |  |  |

**3.5 Phylogenetic trees**

Based on SSR data matrices, phylogenetic trees constructed using graphical analysis in Excel~~,~~ grouped the inbred lines into two distinct categories (Figure .3). Combined with their gene action reports, this classification provides crucial insights for identifying the best crosses to advance in disease-resistance breeding programs. The 23 genotypes were divided into two major groups. Group I comprised seven genotypes, including KALRO lines, their crosses, and lines from CIMMYT. These genotypes lacked amplification of the GLS resistance region during PCR analysis, indicating the absence of the targeted resistance gene. In contrast, Group II formed the larger group, consisting of 17 genotypes. These genotypes exhibited GLS resistance in field evaluations and showed amplification during PCR analysis, confirming the presence of the resistance gene.



**Figure 3: Phylogenetic trees showing banding pattern among the genotypes**

**4 Discussion**

The discovery of a genetic region strongly associated with gray leaf spot resistance, located at approximately 164 base pairs (bp) underscores the utility, highlighting SSR markers' effectiveness in identifying resistance-associated alleles. Thus, offering a valuable tool for genetic analysis and breeding programs aimed at enhancing on improving GLS resistance in maize populations (Hu *et al*., 2024). This region likely corresponds to a quantitative trait locus (QTL) or the genomic region that harbours a GLS resistance gene to GLS in maize. The accurate amplification of this region using simple sequence repeat (SSR) markers underscores their reliability and specificity as molecular tools for detecting important resistance alleles in complex genomes. The strong correlation between the phenotypic data and molecular data is evidence for the presence of a common gene among the genotypes (Carbajal *et al*., 2021).

Resistance to gray leaf spot (GLS) is often controlled by multiple genes, quantitative trait loci (QTL) that harbour major- resistance genes (Gupta *et al*., 2023; Omondi *et al*., 2023). The region identified in this study may contain genes encoding resistance-related proteins, such as nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, which play a crucial role in plant defence mechanisms. Previous studies have shown that NBS-LRR proteins are involved in conferring resistance to gray leaf spots in maize (Yang et al., 2022), and enhancing resistance to *Phytophthora infestans* in tomatoes (Jiang et al., 2018). These proteins recognize pathogen effectors and activate defence responses, leading to either localized or systemic resistance. Additionally, this region may also influence the regulation of genes involved in pathogen recognition, signalling pathways, or the production of antifungal metabolites.

The identification of the resistance-associated locus at 164 bp is crucial for breeding programs, as it offers a dependable molecular marker to differentiate between resistant and susceptible genotypes. This allows breeders to identify resistance alleles at the seedling stage leading to a substantial reduction in time and costs associated with field evaluations. The marker's specificity guarantees the precise selection of parental lines for crosses, thereby improving the efficiency of developing GLS-resistant hybrids (Pei *et al*., 2023). The polymorphism observed within this genetic region across genotypes highlights the genetic diversity that underlying. This diversity is crucial for breeding programs aiming to develop durable resistance, as it reduces the risk of resistance breakdown caused by pathogen evolution. Previous studies by Geffersa et al. (2023) on crops and Peressotti et al. (2010) on grapevine downy mildew have shown the importance of deploying resistance genes to disrupt pathogen adaptation and prevent breakdown. By incorporating diverse alleles from the identified region, breeders can create maize varieties with a broad spectrum of resistance to multiple GLS pathogen strains.

The findings pave the way for further research into the genetic and functional characterization of the resistance-associated region. Fine mapping and sequencing efforts could help identify candidate genes and their mechanisms of action, providing deeper insights into the molecular basis of GLS resistance. This information would be invaluable for integrating gene-editing tools, such as CRISPR-Cas9, into breeding pipelines to enhance the precision and durability of resistance traits in maize (Tang *et al.*, 2023). In conclusion, the identification of the genetic region at 164 bp as a marker for GLS resistance represents a significant advancement in the use of molecular tools for breeding. This discovery not only facilitates marker-assisted selection but also provides a foundation for exploring the genetic architecture of disease resistance in maize. This categorization highlights the genetic variation within the population and provides a basis for selecting genotypes with strong GLS resistance for further breeding efforts. Based on phylogenetic tree analysis, 23 inbred lines were classified into two groups. Group I, consisting of seven genotypes, lacked the GLS resistance gene, as shown by both PCR analyses even though they were resistant in the field. Sometimes, resistance in the field may arise from factors other than genetic resistance. This could be as a result of environmental conditions or epigenetic modifications furthermore, it might be due to other adaptive traits or interactions with the environment.

**5 Conclusions**

This study demonstrates the effectiveness of SSR markers in identifying genomic regions linked to gray leaf spot resistance and emphasizes the importance of combining favourable genes to enhance resistance. The segregation of the F2 population and their parents into distinct genetic groups suggests increased gene action from specific parent combinations. The research sheds light on the molecular mechanisms underlying GLS resistance and lays the groundwork for crop improvement. It underscores the genetic diversity within the populations as a valuable resource for enhancing resistance traits and pinpointing potential genes involved in GLS resistance. Further expansion of the study could further validate these findings and uncover additional resistance genes. Enhancing marker-assisted selection and improving breeding programs or the development of GLS-resistant crops. This could ultimately boost agricultural productivity and food security.

**DISCLAIMER**

The authors of this manuscript hereby declare that no generative AI technologies, including text-to-image generators and large language models (such as ChatGPT, COPILOT, etc.), were used in its writing or editing.

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