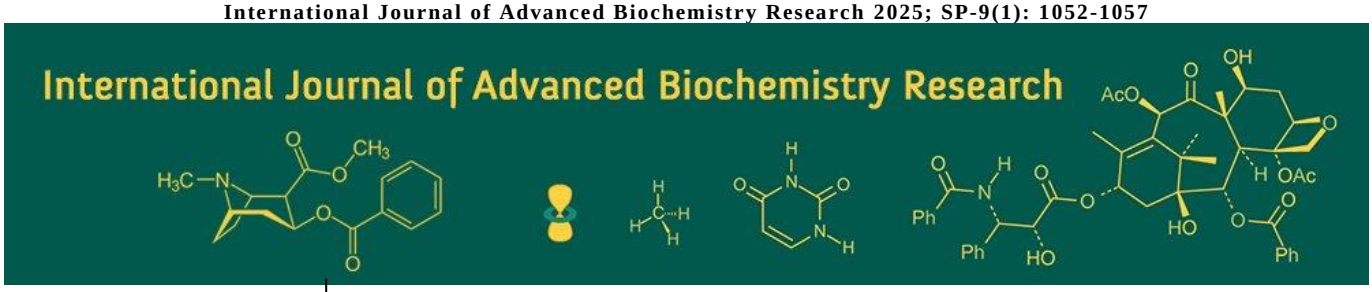

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Genetic diversity studies of maize (*Zea mays* L.) genotypes using SSR markers

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Abstract
Genetic diversity in maize (*Zeamays* L.) plays a key role for future breeding progress. The main objective of our study is to investigate the genetic diversity among 29 CIMMYT maize lines by simple sequence repeat (SSR) markers. In this study, we have characterized 29 maize genotypes at molecular level using 13 primer pairs. The individual genotype has been distinct by constructed dendrogram based upon the dis/similarity coefficient. Among the 13 SSR markers used, one marker has observed to be monomorphic, whereas the rest of the markers revealed polymorphic information content values ranging from 0.067 to 0.499 provided sufficient discrimination to assess the genetic diversity among the genotypes. Hence, we revealed that SSR markers showed to be a powerful tool for detection of genetic diversity in maize genotypes; they can be useful maize for conserving and managing maize germplasm at molecular level.

Keywords: Dendrogram, molecular marker, simple sequence repeat

Introduction
Maize (*Zea mays* L.) is the third most important cereal crop in the world, following rice and wheat. Known as the "Miracle Crop" and the "Queen of Cereals" due to its high productivity potential compared to other members of the Poaceae family, maize has gained global significance with increasing demand from sectors such as food, animal feed, and ethanol production. It is one of the most important food crops globally and serves as a model system for studies in genetics, evolution, and domestication. Genetic diversity is a crucial component in the development of new inbred maize lines and plays a pivotal role in hybrid combinations in maize breeding. It represents a fundamental resource for genetic and breeding studies and is extensively used in hybrid corn production (Anderson and Brown, 1952; Troyer, 2001) [2, 23]. Understanding the relationships between maize breeding lines is essential for parental selection, genetic analysis, and breeding system design. Inbred lines have also been critical for molecular evaluations (Henry and Damerval, 1997; Hou *et al*., 2023) [8, 10].

Knowledge of genetic diversity within maize germplasm ensures that a broad genetic base is maintained, which is vital not only for genetic improvement but also for reducing genetic vulnerability to pests and diseases. Numerous methodologies exist for assessing genetic diversity in maize, but morphological characteristics are often influenced by the environment and may not reliably reflect genetic diversity. Moreover, morphological evaluations are expensive and time-consuming (Dubreuil *et al*., 1996) [6]. In contrast, molecular polymorphisms, which are generally independent of environmental influence, provide a more reliable approach for assessing genetic relationships (Gauthier *et al*., 2002) [7]. Molecular marker analyses offer maize breeders an efficient method for assigning lines to heterotic groups and for selecting parents to develop new hybrids. Among the various molecular markers, simple sequence repeat (SSR) markers are widely regarded as the most suitable for genetic diversity analysis due to their high polymorphism, codominance, repeatability, and reliability (Belalia *et al*., 2019; Rohini *et al*., 2020) [3, 21]. SSR markers, also known as microsatellites, have been frequently employed in genetic diversity studies of maize inbred lines and populations (Matsuoka *et al*., 2002; Warburton *et al*., 2002; Pinto *et al*., 2003) [16, 24, 19]. The diversity observed at the molecular level in maize is of significant importance and has great potential to contribute to future breeding programs (Islam *et al*., 2023) [11].

In this study, we examine the genetic diversity of 29 maize inbred lines using 13 SSR markers, representing the genetic diversity available among currently utilized maize lines.

Materials and Methods

Plant material and DNA extraction

In the present study, 29 maize genotypes were used for diversity analysis were represented in Table 1. The seed materials of these aforementioned genotypes were obtained from Maize Section, Bihar Agricultural University, Sabour. Genomic DNA was isolated from the young leaves of 29 maize genotypes using the SDS-potassium acetate method as described by Hosaka (2004) [9], with minor modifications. The extracted DNA was dissolved in 60 µl of sterile water, and 2 µl of this DNA was used for polymerase chain reaction (PCR). PCR amplification was performed in a total volume of 12 µl, consisting of 2 µl of genomic DNA, 1.2 µl of 10X PCR buffer, 0.1 mM dNTP mix, 1 µM each of forward and reverse primers, and 1 U of Taq DNA polymerase. The thermal cycling conditions included an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 30 s at 94 °C, 40 s at the appropriate annealing temperature, 30 s at 72 °C, and a final extension at 72° C for 10 min, with a hold at 4 °C for 2 min (Kumar *et al*., 2018) [14]. After amplification, the PCR products were separated on 2% (w/v) agarose gel, stained with ethidium bromide, and visualized using a gel documentation system (Bangalore Genei).

Primers

The forward and reverse primer sequences, as well as the annealing temperatures of the 13 SSR markers were obtained from previous studies by Ranatunga *et al*., (2009) [20], Kumar *et al*., (2016) [13], and Kumari *et al*., (2018) [14]. The specific primers used for this analysis are presented in Table 2. These primers were selected to ensure effective amplification and reliable detection of genetic diversity among the maize genotypes.

Diversity analysis

The amplification profiles generated by SSR markers were analyzed using a binary matrix, where the presence of an allele was scored as "1" and its absence as "0". This 1/0 matrix was used to evaluate the molecular similarity of the 29 maize genotypes through Jaccard's similarity coefficient values, calculated using the Dendro UPGMA web-program (http://genomes.urv.cat/UPGMA/).

Based on these similarity values, a dendrogram was constructed using the Phylogeny. fr web-program (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi) to visually represent the genetic relationships among the genotypes. Additionally, the polymorphism information content (PIC) values for the 13 SSR markers were calculated as per Chattopadhyay *et al*., (2008) [4], using the formula: PIC = 1/n Σ 2F(1-F), where *n* represents the number of alleles produced by a marker and *F* is the frequency of a specific allele across the genotypes. These PIC values serve as indicators of the markers' ability to reveal genetic diversity within the population.

Results and Discussion

Thirteen SSR primers were used to assess molecular diversity and interrelationships among 29 maize genotypes. All primers generated clear, polymorphic banding patterns, except for one primer (phi062), which produced a monomorphic profile. A total of 34 alleles were detected, with one monomorphic allele. One primer amplified a 167 bp allele that was present in all genotypes. The number of alleles per primer ranged from 2 to 5, with an average of 2.61 alleles per locus. Among the polymorphic markers, six produced two alleles each, four produced three alleles each, and phi062, phi053, and phi014 produced 1, 4, and 5 alleles, respectively (Fig.1). The number of alleles varied among the genotypes was conformity with Nepolean *et al*., (2013) [17] reported 111 polymorphic alleles with an average of 3.17 alleles per locus; Kumar *et al*., (2016) [13] found 59 alleles with an average of 2.62 per locus; Patel *et al*., (2017) [18] detected 76 alleles with an average of 4.47 per locus. Polymorphism information content (PIC) values ranged from 0.067 (phi029) to 0.499 (phi065), which is lower than values reported by Ranatunga *et al*., (2009) [20], Adeyemo *et al*., (2011) [1], Sserumaga *et al*., (2013) [22] and Patel *et al*., (2017) [18]. However, the results are consistent with Kumar *et al*., (2016) [13], who reported PIC values ranging from 0.142 to 0.497 using the same set of primers. The highest PIC values were observed for primers phi065 (0.499) followed by primer phi064 (0.452), while the lowest PIC values were for phi029 (0.067). These PIC values highlight the effectiveness of the SSR loci in detecting genetic differences among 29 maize genotypes. The primers phi065 and phi064 were identified as the most informative markers for genetic diversity analysis due to their high PIC values. The lower allelic diversity and PIC values observed in this study may be attributed to the narrow genetic base of the 29 maize genotypes or the use of tri, tetra, and penta nucleotide primers.

Cluster Analysis

The dendrogram of 29 maize genotypes was constructed using 33 polymorphic loci generated by 13 SSR markers. The similarity coefficients among the genotypes ranged from 0.20 to 0.938, reflecting varying degrees of genetic relatedness (Table 4). The highest similarity coefficient (0.93) was recorded between genotypes G2 and G3, indicating a high degree of genetic similarity, possibly due to common parentage. Conversely, the lowest similarity coefficient (0.20) was observed between genotypes G9 and G23, suggesting substantial genetic divergence, which could be attributed to differences in morphological traits, geographical origins, or distinct parental lines.

The phylogenetic analysis grouped the 29 maize genotypes into two main clusters, I and II, with further subdivision into sub clusters Ia and Ib (Fig. 2). Cluster Ia consisted of five inbred lines (G1, G2, G3, G7, G14), while cluster Ib included three lines (G4, G9, G22). Cluster II encompassed the majority of the genotypes, *viz*., G28, G27, G24, G17, G25, G16, G15, G11, G23, G26, G29, G13, G10, G21, G20, G18, G12, G19, and G8. Interestingly, genotypes G5 and G6 formed a unique cluster, indicating their distinct genetic background. The clustering pattern suggests significant genetic variability among the genotypes, with the most genetically diverse genotypes being potential candidates for use in breeding programs to enhance genetic diversity. The results of this study are consistent with previous reports of Ranatunga *et al*., (2009) [20] classified 45 maize genotypes into two major clusters, which were further divided into several sub-clusters. Similarly, Kanagarasu *et al*., (2013) [12] identified five major clusters among 27 maize inbred lines

using ten SSR markers. Patel *et al*., (2017) [18] grouped eight genotypes into three divergent clusters based on similarity coefficients, while Kumar *et al*., (2016) [13] reported five clusters among 13 genotypes. These findings highlight the presence of genetic variability in maize genotypes, which is essential for developing new maize varieties with enhanced agronomic traits.

These results suggest that SSR markers can effectively characterize maize genotypes at the molecular level, assisting breeders in assigning lines to heterotic groups and selecting parents for hybrid development. Moreover, SSR markers can distinctly identify individual genotypes through unique allelic patterns, a key application in varietal protection. Based on molecular marker analysis, seven distinct inbred lines CML 164 (G2), CML 411 (G3), CML 305 (G4), CML 304 (G5), CML 25 (G7), CML 306 (G28), and CML 307 (G29) were identified as suitable for cross combinations. The findings provide valuable insights for future maize breeding programs, enabling breeders to select appropriate loci for maintaining genetic diversity, ensuring seed purity, and developing new hybrids through strategic introductions, crosses, and selections.

Table 1: List of Twenty-nine maize genotypes used for genetic diversity studies

S. No.	Entry	Codes	Origin
1	CML50	G1	CIMMYT
2	CML164	G2	CIMMYT
3	CML411	G3	CIMMYT
4	CML305	G4	CIMMYT
5	CML304	G5	CIMMYT
6	CML19-1-B	G6	CIMMYT
7	CML25-1-B	G7	CIMMYT
8	CML116-1-BB	G8	CIMMYT
9	CML171-BBB	G9	CIMMYT
10	CML18	G10	CIMMYT
11	CML27-2-BBB	G11	CIMMYT
12	CML-28-3-B	G12	CIMMYT
13	CML33-1-BB	G13	CIMMYT
14	CML474	G14	CIMMYT
15	CML70-2-BBB	G15	CIMMYT
16	CML73-4-B	G16	CIMMYT
17	CML118	G17	CIMMYT
18	CML130-2-BBB	G18	CIMMYT
19	CML139	G19	CIMMYT
20	CML159-1-B	G20	CIMMYT
21	CML162	G21	CIMMYT
22	CML165	G22	CIMMYT
23	CML172-BB	G23	CIMMYT
24	CML189-B	G24	CIMMYT
25	CML308-1-BBB	G25	CIMMYT
26	CML328-2-BB	G26	CIMMYT
27	CML451	G27	CIMMYT
28	CML306	G28	CIMMYT
29	CML307-1-BBB	G29	CIMMYT

Table 2: List of SSR markers used in the genetic diversity of maize genotypes

Markers	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp.(ºC)
ngl 391	CAGATATCACAGCATCAGAAGATCA	AAAATGTAAGAACTTGTTTGGGATT	56
phi 011	TGTTGCTCGGTCACCATACC	GCACACACACAGGACGACAGT	60
nc 133	AATCAAACACACACCTTGCG	GCAAGGGAATAAGGTGACGA	54
phi 056	ACTTGCTTGCCTGCCGTTAC	CGCACACCACTTCCCAGAA	56
phi 014	AGATGACCAGGGCCGTCAACGAC	CCAGCTTCACCAGCTTGCTCTTCGTG	52
phi 029	TTGTCTTTCTTCCTCCACAAGCAGCGAA	ATTTCCAGTTGCCACCGACGAAGAACTT	56
phi 032	CTCCAGCAAGTGATGCGTGAC	GACACCCGGATCAATGATGGAAC	56
phi 034	TAGCGACAGGATGGCCTCTTCT	GGGGAGCACGCCTTCGTTCT	56
phi 053	CTGCCTCTCAGATTCAGAGATTGAC	AACCCAACGTACTCCGGGCAG	56
phi 062	CCAACCCGCTAGGCTACTTCAA	ATGCCATGCGTTCGCTCTGTATC	56
phi 063	GGCGGCGGTGCTGGTAG	CAGCTAGCCGCTAGATATATACGCT	54
phi 064	CCGAATTGAAATAGCTGCGAGAACCT	ACAATGAACGGTGGTTATCAACACGC	56
phi 065	AGGGACAAATACGTGGAGACACAG	CGATCTGCACAAAGTGGAGTAGTC	54

Table 3: Summary of performance of the SSR markers used in the present study

Markers	Alleles	Rare alleles	Genotypes with multiple alleles	Size range (bp)	Highest frequency allele		PIC value
					Size (bp)	Frequency	
phi011	2	1	0	220-234	133	51.7	0.411
phi029	2	1	0	148-155	148	96.5	0.067
phi034	3	0	3	105-135	121	51.7	0.450
phi056	3	0	0	232-250	244	68.9	0.317
phi062	1	0	0	NA	167	100	0.000
phi065	2	0	0	150-160	150	51.7	0.499
bnlg391	3	0	0	120-152	133	51.7	0.411
phi014	5	2	6	147-334	165	89.6	0.166
phi032	2	0	20	175-246	246	96.5	0.233
phi053	4	1	0	108-225	188	48.2	0.313
phi063	3	0	14	135-245	195	62	0.369
phi064	2	0	0	105-119	105	65.5	0.452
nc133	2	0	0	95-120	120	86.2	0.238

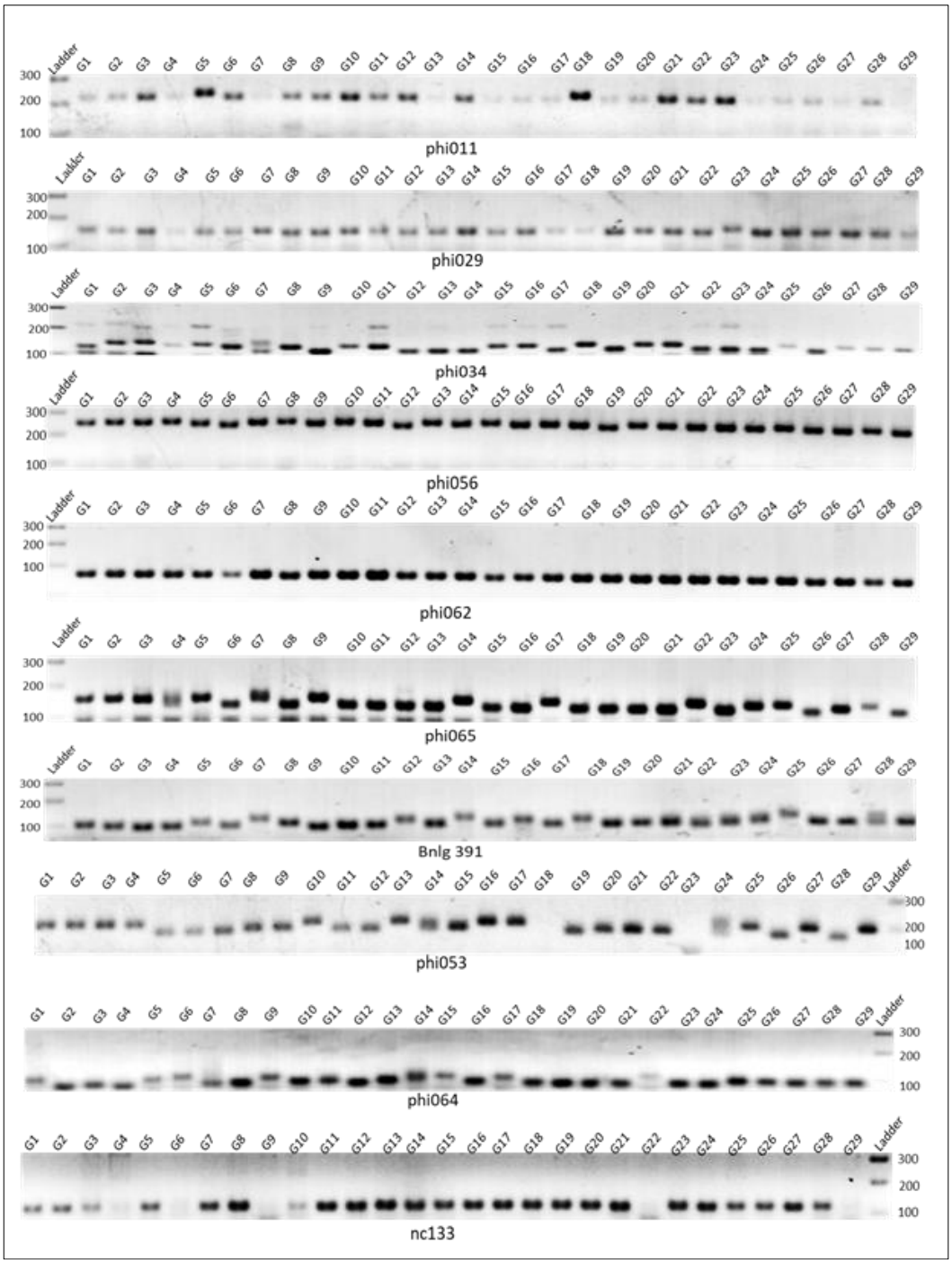

Fig 1: PCR amplification using SSR primers - phi011, phi029, phi034, phi056, phi065 bnlg391, phi014 phi032, phi053, phi064 &nc133 in twenty nine maize genotypes.

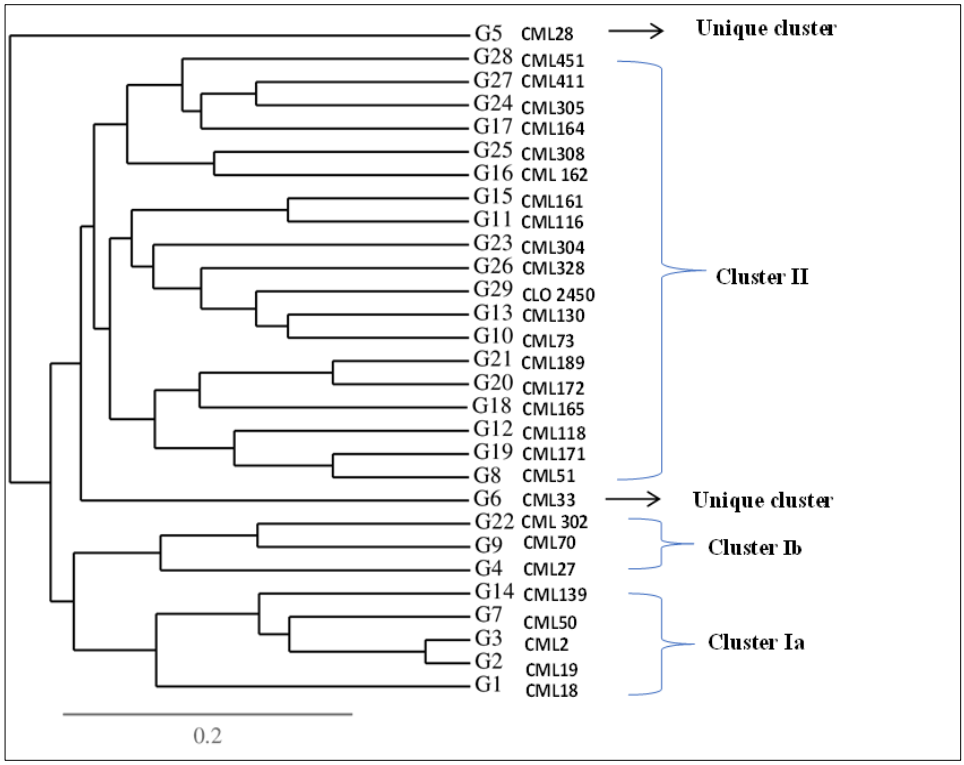

Fig 2: Dendrogram of 29 maize genotypes developed on the basis of polymorphism revealed by the 13 SSR markers.

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