Maize Journal (April 2025) 14(1): 6-13

RESEARCH PAPER

Assessment of molecular diversity among maize inbred lines using SSR markers

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Abstract This study aimed to assess the genetic diversity among 112 maize inbred lines using Simple Sequence Repeat (SSR) markers to support targeted breeding programs and heterotic group classification. Understanding the molecular genetic diversity among and within inbred lines is important for maize improvement in different breeding programs. Only seven primer pairs out of 22 SSR primers used in the study showed clear polymorphism yielding 2-4 alleles per locus with an average of 3.28 alleles per marker. Among the 7 polymorphic markers, bnlg 238, umc 1029, umc 2077 and bnlg 1335 recorded the higher PIC values, the most effective alleles and the higher Shannon's information index and Nei's genetic diversity index confirming their utility for diversity studies. The polymorphism information content (PIC) of the SSR loci ranged from 0.46 to 0.71. PIC was highest for the SSR primer Bnlg 238 (0.71) followed by Umc 1029 and Umc 2077 (0.67) and lowest for the primer Bnlg 1666 (0.46). By using UPGMA cluster analysis, the 112 inbred lines were grouped into three major clusters. Cluster II was the largest with 46 inbred lines followed by cluster I with 39 inbred lines and cluster III with 27 inbred lines. Sub-clusters further diverged at higher similarity coefficients, reflecting fine-scale genetic differences. The SSR markers effectively captured high polymorphism among maize inbred lines, enabling precise genotype identification.The heterotic potential of clusters (e.g., divergent groups I vs. III) can be exploited for hybrid breeding..

Keywords: Inbred • Molecular diversity • Markers • Polymorphism

Introduction

Maize (*Zea mays* L) is one of the most important cereal crops having wider adaptability under varied agro-climatic conditions. Globally, maize is known as queen of cereals as it has the highest genetic yield potential among the cereals. Maize crop is cultivated in an area of 9.8 million hectares, with a production of 37.62 million tonnes and with the productivity of 2394 kg ha^{-1} in India (Indiastat.com, 2016-17). While, in Andhra Pradesh, maize is cultivated in an area of 2.96 lakh ha with a production of 19.84 lakh tonnes and productivity of 6543 kg/ha. (Directorate of Economics and Statistics.ap.gov.in, 2023-24). Maize is used to provide raw materials for industries for a variety of purposes, including maize starch, dextrose, maize syrup and maize flakes.

The study of genetic diversity is fundamental to maize breeding programs, enabling the selection of genetically diverse parents to achieve heterotic hybrids while supporting the characterization and conservation of germplasm. While morphological traits have traditionally been used to assess diversity, they are influenced by environmental factors and often lack precision. In contrast, DNA-based molecular markers provide accurate, reproducible, and environment-independent data, making them indispensable for quantifying genetic variation.

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Received: 28 November 2024/ Accepted: 28 March 2025

Among available molecular markers, Simple Sequence Repeats (SSRs) are particularly valuable due to their high polymorphism (multi-allelic nature), codominant inheritance, ease of detection and genome-wide distribution. These attributes have established SSRs as a gold standard for evaluating genetic diversity in maize populations. By elucidating molecular-level relationships among inbred lines, SSR markers help breeders identify genotypes with maximum divergence to exploit heterosis, classify inbred lines into heterotic groups and select optimal parental combinations for hybrid development. The strategic use of SSR-based diversity analysis can significantly enhance maize productivity by ensuring sustainable utilization of genetic resources. This study employs SSR markers to characterize maize inbred lines, aiming to facilitate targeted breeding for yield improvement and stress resilience.

Materials and methods

The experimental material was evaluated using alpha lattice design with 112 maize inbred lines (Table 1) during *rabi* 2022-23 at Agricultural Research Station, Peddapuram. The whole experimental area was divided into two replications. Each replication consisted of 28 blocks. In each block, 4 inbred lines were allocated and each inbred line was planted in two rows each of 4 meter in length with spacing of 60 cm between rows and 20 cm within row. All the recommended package of practices and need based plant protection measures were adopted throughout

Table 1. List of Maize inbred lines used in the study

S.No.	Inbred lines	S.No.	Inbred lines	S.No.	Inbred lines	S.No.	Inbred lines
1.	PL 22424	29.	PL 22404	57.	PL 22391	85.	PI 416
2.	PL 22429	30.	PL 22416	58.	PL 22412	86.	PI UK
3.	PL 22394	31.	PL 22402	59.	PL 22415	87.	PI 417
4.	PL 22396	32.	PI394	60.	PL 22405	88.	PI 418
5.	PL 22436	33.	PI 395	61.	PL 22423	89.	PI 419
6.	PL 22444	34.	PI 1	62.	PL 22439	90.	PI 420
7.	PL 22440	35.	PI 397	63.	PL 22409	91.	PI 421
8.	PL 22389	36.	PI 398	64.	PL 22441	92.	PI 422
9.	PL 22408	37.	PI 399	65.	PL 22411	93.	PI 423
10.	PL 22426	38.	PI 400	66.	PL 22437	94.	PI 424
11.	PL 22449	39.	PI 401	67.	PL 22430	95.	PI 425
12.	PL 22445	40.	PI 402	68.	PL 22450	96.	PI 426
13.	PL 22422	41.	PI 403	69.	PL 22442	97.	PI 427
14.	PL 22428	42.	PI 404	70.	PL 22447	98.	PI 428
15.	PL 22431	43.	PI 405	71.	PL 22410	99.	CML 72
16.	PL 22434	44.	PL 22435	72	PL 22446	100.	UMI 1200
17.	PL 22401	45.	PL 22398	73.	PL 22403	101.	CML 425
18.	PL 22427	46.	PL 22395	74.	PL 22438	102.	CML 451
19.	PL 22421	47.	PL 22414	75	PI 406	103	CML 474
20.	PL 22407	48.	PL 22413	76.	Pi 407	104.	CML 581
21.	PL 22390	49.	PL 22425	77.	PI 408	105.	CML 582
22.	PL 22392	50.	PL 22420	78.	PI 409	106.	CL 02450
23.	PL 22397	51.	PL 22400	79	PI 410	107.	LM 13
24.	PL 22448	52.	PL 22406	80.	PI 411	108.	LM 14
25.	PL 22399	53.	PL 22433	81.	PI 412	109.	BML 6
26.	PL 22393	54.	PL 22443	82.	PI 413	110	BML 7
27.	PL 22417	55.	PL 22419	83.	PI 414	111.	BML 45
28.	PL 22432	56.	PL 22418	84.	PI 415	112.	CAL 1812

the crop growth period to raise a good crop.

Observations on different parameters *viz*., days to 50% anthesis, days to 50% silking, Anthesis silking interval, plant height (cm), tassel height (cm), tassel extrusion, tassel size and yield parameters like ear height (cm), ear position, ear length (cm), ear girth (cm), days to maturity, kernel rows per ear, number of kernels per row, shelling percentage, 100-kernel weight (g), ear yield per plant (g) and kernel yield per plant (g) were recorded on five plants selected at random from each entry in each replication.

The SSR primers (Table 2) were selected based on the earlier reports of Kumar *et al*. (2022) and Kiran (2016) are used to study the diversity analysis of maize inbred lines in the present study. DNA was extracted from the leaf tissue collected at 15 days after sowing from all the inbred lines using the method described by Zheng *et al*. (1995) and Vivekananda *et al*. (2018) with certain modifications, following CTAB extraction method. Out of 22 primers, 7 have showed clear polymorphism. Information regarding the 22 SSR primers given in Table 1. PCR was carried for the reaction mixture and the polymerase chain reaction (PCR) was carried out as per the standard procedure. The amplified products of PCR were resolved on 3 % agarose gel. A 50 bp ladder was used for sizing of the products. Electrophoresis was done at 80-100 volt for 2 h in 1X TAE buffer and bands were visualized and documented in gel documentation system. The SSR markers generated clear, unambiguous bands of varying molecular sizes, which were scored based on allele number and polymorphism. The allele number shows count of distinct alleles per locus reflecting genetic variability while, the polymorphism depicts differences in amplicon base pair (bp) lengths among genotypes. The scoring approach enables precise quantification of genetic diversity as SSR markers exhibit high reproducibility and sensitivity to allelic variations.

Results and discussion

In this study, 112 maize inbred lines were screened for genetic diversity using 22 simple sequence repeat (SSR) markers and the results are discussed hereunder. The bands that were clear and unambiguous were scored. Bands were scored (1) for presence and (0) for absence in all 112 inbred lines for all markers. Only seven out of 22 primer pairs showed clear polymorphism. The number of bands produced with the 7 primer pairs were 23. The markers yielded two to four alleles with an average of 3.28 alleles per marker. The size of the bands varied from 92 bp to 460 bp.

The Polymorphic information content (PIC) was taken as the measure to detect the polymorphism of a marker locus and genetic diversity evolution. The range of PIC values was 0.46 to 0.71 (Table 3). PIC value was the highest for the SSR primer bnlg 238 (0.71), followed by umc 1029 and umc 2077 (0.67) while, it was the lowest for the primer bnlg1666 (0.46). The overall mean of PIC was 0.6 (Table 3). The higher the PIC value, the more informative the SSR marker. Hence, the primers (bnlg 1335, umc 1029, umc 2077, bnlg 2086, bnlg 238, phi 054) with PIC values greater than 0.5 were found to be highly informative. The primer pair (bnlg 1666) recorded the PIC value of more than 0.45 but less than 0.5 indicating their moderate utility in providing the genetic diversity information. Similar PIC results were also reported by Salami *et al.* (2016) with a mean PIC value of 0.71; Wende *et al*. (2018) with a mean PIC of 0.54; Adu *et al*. (2019) with a mean PIC of 0.68; and Kumar *et al.* (2022) with a mean PIC of 0.45. These high PIC markers can be used for diversity studies and evolution.

The number of equally frequent alleles needed to achieve the same expected heterozygosity is known as the effective number of alleles. Effective allele numbers should always be less than or eventually equal to observed allele numbers. The number of effective alleles in the current study ranged from 1.7 (bnlg 1666) to 3.3 (bnlg 238) with a mean value of 2.42. The higher number of effective alleles were found in bnlg 238 (3.3) and umc 1029 (2.9) while, the lower number of alleles were found in bnlg 1666 (1.7) and bnlg 2086 (1.8). This suggests that these markers could be potentially used for molecular characterization of maize inbreds from various sources. The results are in accordance with Jyothi sahu *et al.* (2023) and Mathiang *et al*. (2022).

The Number of observed alleles per locus (Na*) in this study was 23 alleles. The marker, bnlg 238, umc1029 and phi054 produced 4 allele per locus while, markers umc 2077, bnlg 2086 and bnlg 1335 generated 3 allele per locus with an average of 3.28 allele per locus indicating the usefulness of these markers in diversity studies (Plate 1 to 6). Results of similar nature were earlier reported by Mathiang *et al*. (2022). Nei's genetic diversity index measures the amount of genetic variation or average

Table 2. List of SSR primers used for PCR amplification in the present investigation

S.No.	Primer	Bin loc	F/R	Sequence	T_a
1	Bnlg 198	2.08	F	GTTTGGTCTTGCTGAAAAATAAAA	50.9
			R	GCTGGAGGCCTACATTATTATCTC	54.8
2	Bnlg 1335	2.08	F	GAAGGTTGCTCTTCCACTGG	55.8
			R	TGGTTTGTGCAAGTGTCACC	56.1
3	Umc 2210	8.05	F	GATGCTACCATTTCAGTGAGCGAT	57.2
			R	AGCGGGTCGATCTTTCTCTTAGTT	57.7
4	Umc 1665	8.05	F	CAATCAGGAGCCAGGGAGATG	57.5
			R	CTTAAACTTGTCGAGACGGTCCTG	57.3
5	Umc 1029	7.04	F	AACACCTGCTGGATATGGATCACT	58.0
			R	GGAAGAAAAATGTCGACCTGCTC	56.2
6	Bnlg 1666	7.04	F	GCTGGTAGCTTTCAGATGGC	56.2
			R	TGTCCCTCCTCCAGTTTCAC	56.5
7	Bnlg 240	8.06	F	AAGAACAGAAGGCATTGATACATAA	52.6
			R	TGCAGGTGTATGGGCAGCTA	58.8
8	Umc 1728	8.06	F	AGTACTTTCAGGCAGGGACCTTCT	59.6
			R	AACGCACTTCTTGTAGCTGTAGGG	58.9
9	Phi 056	1.01	F	ACTTGCTTGCCTGCCGTTAC	58.5
			R	CGCACACCACTTCCCAGAA	57.9
10	Umc 1293	10.01	F	GTATCCGTTTCTCATGCAACACAC	56.4
			R	GATCTCGATCTGCTTCATCATCTG	55.1
11	Umc 2077	2.09	F	AAACTCACTGAACATGATCCTGGC	54.8
			R	CTGGTTCGGATGCAAGTAGTCAG	56.6
12	Umc 1086	4.08	F	CATGAAAGTTTTCCTGTGCAGATT	65.0
			R	GGGCAACTTTAGAGGTCGATTTATT	67.7
13	Umc 1559	4.09	F	CTTGCTAGAGTCGGTGAACAACAA	56.1
			R	AACCAAGCTCCTTAATGAGGTCAC	56.9
14	Umc 1644	3.06	F	CCATAAACTGTTCCTTTGGCACAC	58.3
			R	CTTTCACGTGTTAAGGGAGACACC	56.2
15	Umc 2169	3.06	F	ACTACTCCTCGGATAGCCACG	53.7
			R	GACGAGTAGAGGCTCTGGGAC	56.0
16	Bnlg 2086	1.04	F	CGGAACCTGCTGCAGTTAAT	55.9
			R	GAGATGCAGGAATGGGAAAA	53.7
17	Phi 330507	5.04	F	GTAAAGTACGATGCGCCTCCC	58.0
			R	CGGGGTAGAGGAGAGTTGTG	56.9
18	Umc 1568	1.02	F	AAGTCCAGCCAAGTTCATCAAAGA	56.8
			R	ACTGTAACTAAACTGGGTGTGCCC	59.0
19	Nc 003	2.06	F	ACCCTTGCCTTTACTGAAACACAACAGG	61.0
			R	GCACACCGTGTGGCTGGTTC	61.8
20	Phi 085	5.06	F	AGCAGAACGGCAAGGGCTACT	61.3
			R	TTTGGCACACCACGACGA	57.5
21	Bnlg 238	6.00	F	CTTATTGCTTTCGTCATACACACACATTCAT	57.7
			R	GAGCATGAGCTTGCATATTTCTTGTGG	58.5
22	Phi 054	10.03	F	AGAAAAGAGAGTGTGCAATTGTGATAGAG	60.8
			R	AATGGGTGCCTCGCACCAAG	56.9

T_a – Annealing temperatures; F – Forward sequence; R – Reverse sequence; Bin Loc – Bin Location

Table 3. Number of alleles and polymorphic information content (PIC) value of the seven polymorphic SSR markers used in the present study

No.	SSR Marker	Na*	Ne*	MAF	I*	PIC	Nei*	Amplicon sizerange (bp)
1	bnlg1335	3	2.8	0.40	1.06	0.64	0.64	99 to 246
2	umc1029	4	2.9	0.44	1.19	0.67	0.66	110 to 460
3	bnlg1666	2	1.7	0.70	0.61	0.46	0.42	97 to 200
4	umc2077	3	2.5	0.50	1.00	0.67	0.60	140 to 320
5	bnlg2086	3	1.8	0.70	0.8	0.51	0.46	92 to 200
6	bnlg238	4	3.3	0.40	1.28	0.71	0.70	150 to 250
7	phi054	4	1.94	0.68	0.92	0.54	0.48	95 to 210
	Minimum	2	1.7	0.40	0.61	0.46	0.42	92
	Maximum	4	3.3	0.70	1.19	0.71	0.70	460
	Total	23	16.9	3.82	6.86	4.2	3.96	
	Mean	3.28	2.42	0.55	0.98	0.6	0.56	

Na* = Number of alleles; Ne* = Number of effective alleles; MAF = Major allelic frequency; I* = Shannon's information index; PIC = Polymorphic information content; Nei** = Genetic diversity index; bp = Base pairs

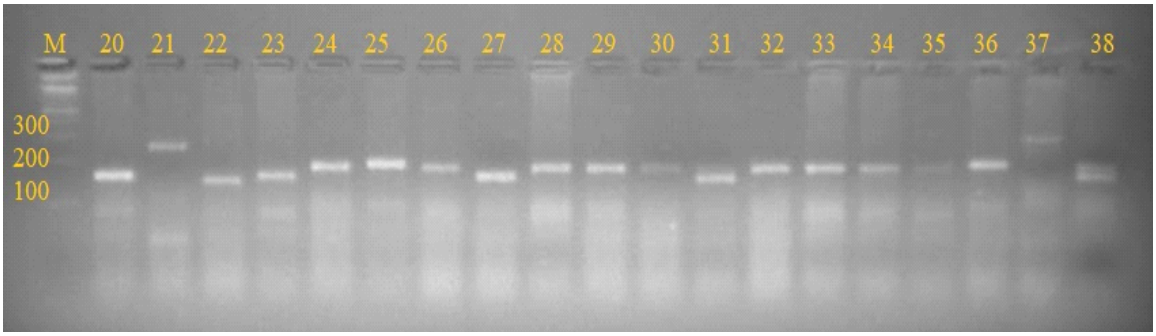

Plate 1. SSR marker profiles of generated by primer umc 2077

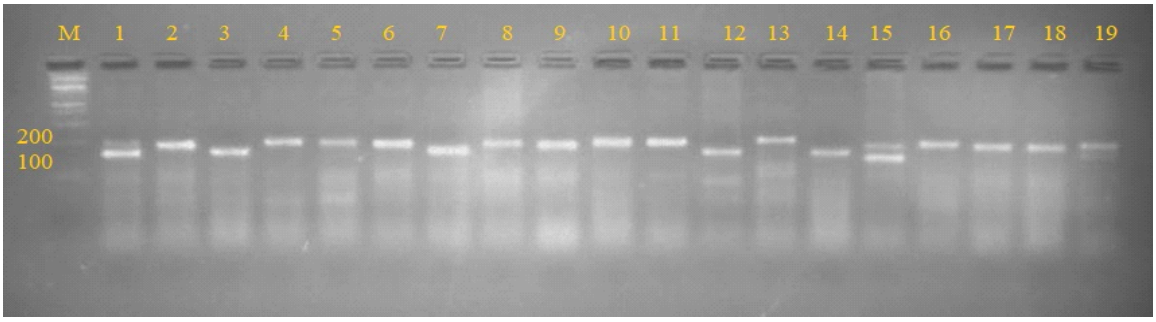

Plate 2. SSR marker profiles generated by primer bnlg 238

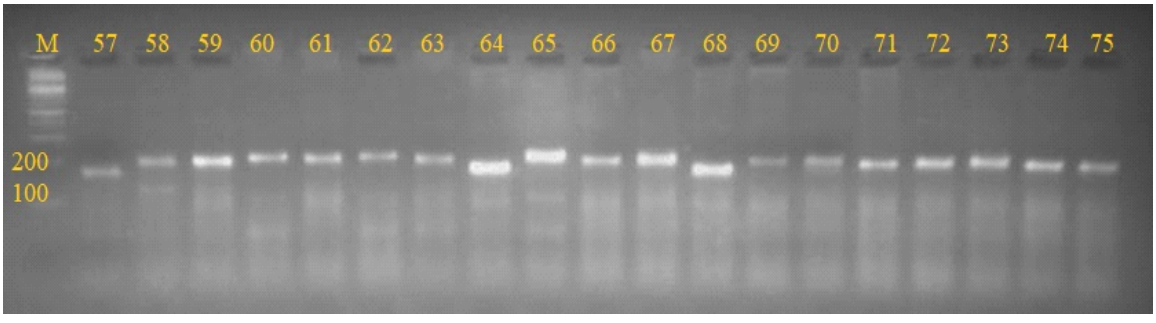

Plate 3. SSR marker profiles generated by primer bnlg 1335

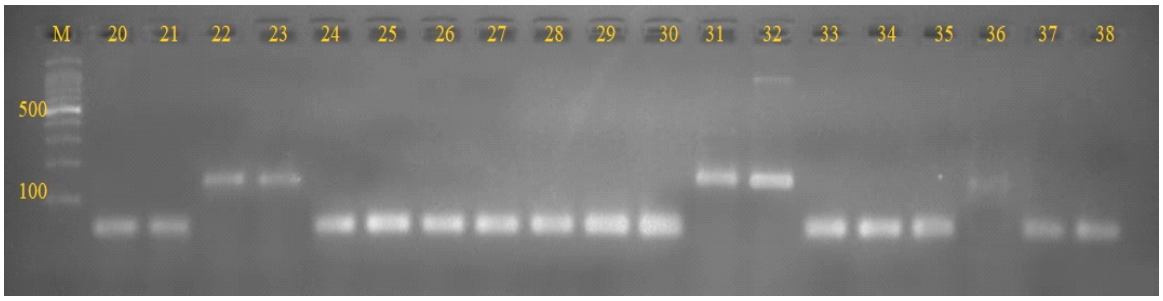

Plate 4. SSR marker profiles generated by primer bnlg 1666

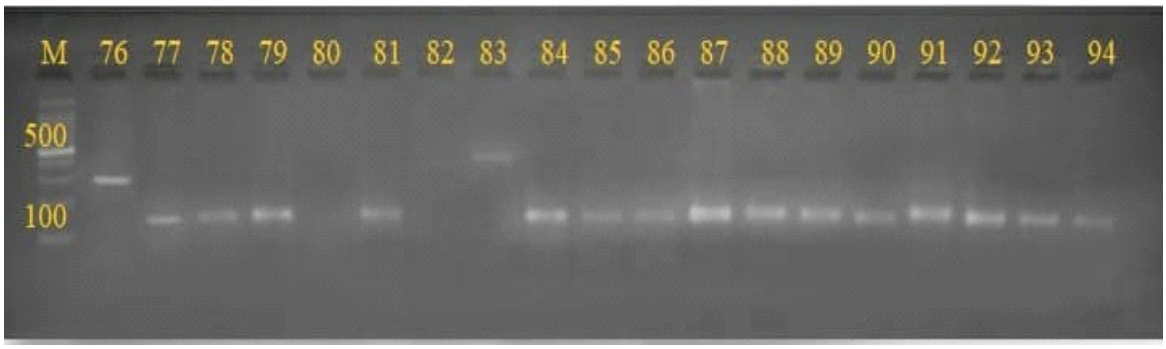

Plate 5. SSR marker profiles generated by primer umc 1029

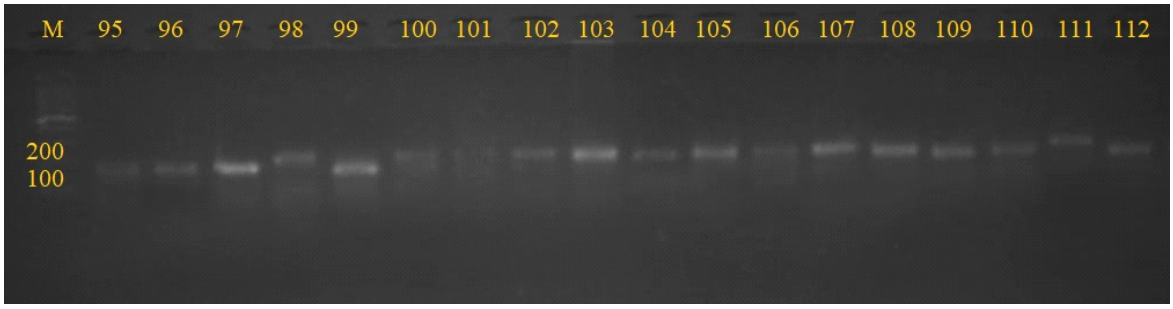

Plate 6. SSR marker profiles generated by primer phi 054

Table 4. Clustering pattern based on UPGMA dendrogram in 112 inbred lines of maize (*Zea mays* L.)

Cluster	Sub cluster	Number of inbred lines	Name of inbred lines
I	IA	36	PL 22439, PL 22405, PL 22415, PI 411, PL 22412, PI 415, PI 414, PI 413, PL 22402, PI 410, PI 422, PI 418, PL 22441, PL 22420, PL 22430, PI 406, PL 22437, PI 421, PL 22447, PI 395, PI 408, PI 407, PI 419, PL 22411, PL 22409, PL 22442, PL 22450, PI 424, PI 418, BML 7, PI 425, PL 22438, PL 22446, PL 22391, CML 72, PI 427
	IB	3	PL 22425, PL 22431, BML 6
II	IIA	26	CAL 1812, PL 22429, BML 45, LM 13, PL 22410, CML 474, PI 426, CL02450, UMI 1200, CML 582, CML 425, CML 581, PL 22414, LM 14, PI 417, LM 14, PI 416, PI 428, PL 22389, PL 22403, PL 22423, PI 406, PL 22436, PL 22394, PL 22444, PL 22428, PL 22424
	IIB	20	PI 403, PI 401, PI 400, PL 22421, PL 22408, PL 22416, PL 22396, PL 22428, PL 22427, PL 22401, PL 22413, PL 22395, PI 398, PL 22449, PL 22422, PL 22434, PI 395, PI 402, PI 394, PL 22440
III	IIIA	20	PL 22393, PL 22397, PL 22399, PL 22417, PL 22432, PL 22404, PL 22448, PL 22433, PL 22406, PL 22400, PL 22443, PL 22435, PI 404, PI 405, PL 22420, PI 397, PI 1, PI 399, PL 22392, PL 22407
	IIIB	7	PL 22419, PL 22390, PL 22418, PL 22398, PL 22402, PL22445, PL 22426

genetic diversity that exists at each locus. The marker bnlg 238 (0.70) recorded the higher level of genetic diversity while, marker bnlg 1666 (0.42) recorded the lower level of genetic diversity with an average value of 0.56. Mathiang *et al*. (2022) and umar *et al.* (2022) reported similar results.

The genetic similarity matrix was computed using Jaccard's coefficient and analyzed through UPGMA (Unweighted Pair Group Method with Arithmetic Averages) in DARwin 6.0 software. This hierarchical clustering approach grouped the 112 maize inbred lines based on their SSR marker profiles. The UPGMA analyses revealed three major groups cluster I, II and III (Table 4 and Figure 1). Cluster II was the largest with 46 inbred lines followed by cluster I with 39 inbred lines and cluster III with 27 inbred lines. Cluster I was further separated into two sub-clusters, IA and IB with 36 and 3 inbred lines, respectively. Cluster II was further subdivided into two sub-clusters, IIA and IIB, with 26 and 20 inbred lines, respectively. Cluster III was further subdivided into two sub-clusters, IIIA and IIIB, with 20 and 7 inbred lines, respectively. At higher similarity coefficients, these sub-clusters further differentiated into distinct sub-sub clusters, revealing fine-scale genetic relationships among the inbred lines. Despite number of groups, the clustering pattern obtained in this study was like the clustering patterns reported by Sharma *et al*. (2018) with inbred lines classified into four sub clusters and grouping of fourth sub cluster to two sub cluster, Ranatunga *et al*. (2009) classified inbred lines into two distinct clusters; Kumar *et al.* (2022) identified five distinct clusters, Kanagarasu *et al*. (2013) classified inbred lines into five clusters and Kumar *et al*. (2016) classified inbred lines into five clusters, Mukri *et al*. (2022) classified inbred lines into three clusters based on the UPGMA dendrogram. These variations likely reflect differences in germplasm sets, marker selection and environmental adaptations of the studied materials. Identifying genetic sub-populations provides crucial insights for breeding programs, enables strategic crossing between divergent clusters to maximize heterosis, facilitates germplasm conservation by identifying unique genetic resources, genetic studies, improves accuracy of genome-wide association studies (GWAS) by controlling population structure (Bryc *et al*., 2010), enhances understanding of ecological adaptation and evolutionary relationships (Glover *et al*., 2012; Moore *et al*., 2014).

Conclusion

This study demonstrated the effectiveness of Simple Sequence Repeat (SSR) markers as a powerful tool for assessing genetic diversity among maize inbred lines, with direct applications in hybrid breeding programs. Only 7 of 22 SSR primers (32%) showed clear polymorphism generating 2-4 alleles with a mean of 3.28 alleles per marker. Polymorphism Information Content (PIC) values ranged from 0.46 to 0.71. Four markers *viz*., bnlg 238,

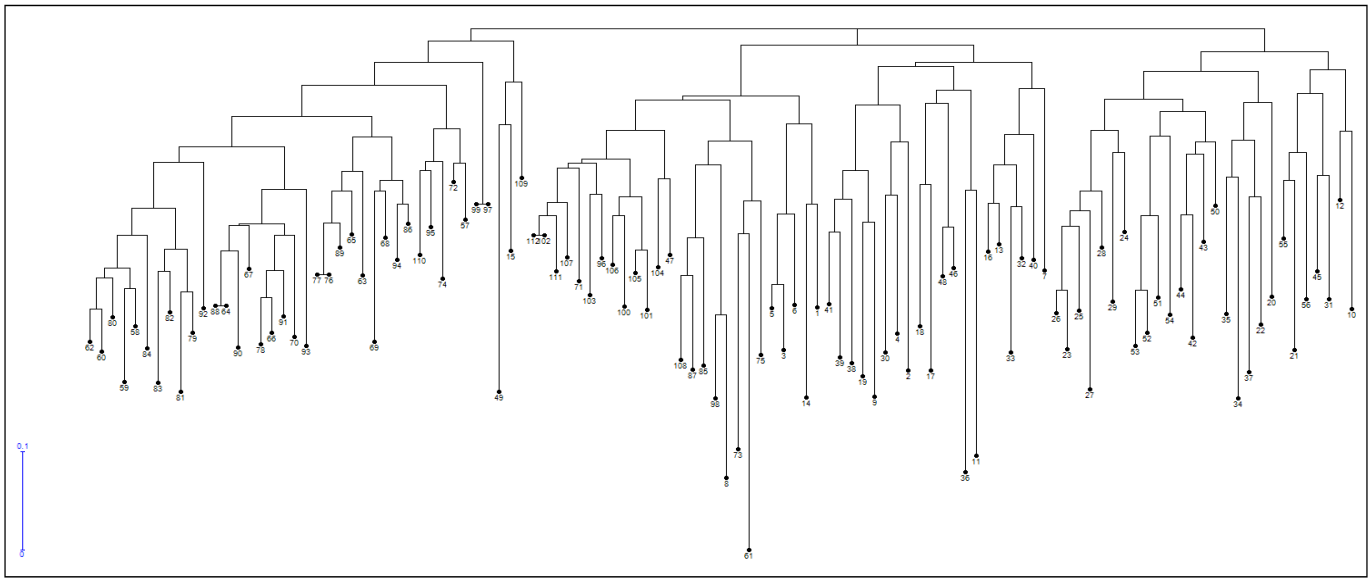

Figure 1. UPGMA dendrogram generated based on Jaccard's similarity index using SSR markers showing variability among 112 inbreds lines of maize (*Zea mays* L.)

umc 1029, umc 2077, and bnlg 1335 proved particularly informative, showing highest PIC values (0.67-0.71), elevated shannon's information index, strong Nei's genetic diversity index. UPGMA cluster analysis (Jaccard's coefficient) revealed three major clusters among 112 inbred lines with substantial genetic variation between clusters revealing potential heterotic groups for hybrid development. The identified polymorphic markers are valuable for germplasm characterization, parental line selection and heterotic group formation. Clustering of genotypes provided a framework for strategic crossing between divergent groups maximizing heterosis in hybrid development. These findings contribute significantly to maize breeding efforts by validating SSR markers for diversity assessment, identifying optimal markers for future studies and provided a genetic basis for hybrid breeding strategies. The genetic information generated will be particularly valuable for developing high-yielding maize hybrids through informed selection of genetically diverse parental lines.

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