CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Molecular diversity, population structure analysis, and assessment of parent hybrid relationships in fodder maize

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Abstract: *Maize is considered one of the most important cereal fodder crops. Many studies on morphological diversity in fodder maize have been helpful in obtaining good heterotic hybrids. The current study focused on analysing diversity of 28 fodder maize inbreds with 30 SSR markers, which revealed total of 110 alleles; and their polymorphic information content (PIC) values ranged from 0.064 to 0.745. Population structure analysis revealed four subpopulation groups with the ΔK value of 132.70. Clustering based on the pairwise dissimilarity coefficient grouped the genotypes into two major and four sub-clusters. The high dissimilarity (0.777) observed between DM 84 and UMI 1221 indicated that these two were highly divergent. Principal coordinate analysis also showed diverse nature of inbreds and corroborated the clustering pattern. Parental diversity and their heterosis performance revealed that parents with average or narrow divergence could be useful in obtaining hybrids with medium/early flowering and moderate/high crude protein content.*

Keywords: *Diversity, fodder maize, hybrids, SSR, population structure*

INTRODUCTION

Maize (*Zea mays* L.) is used as a staple food crop in most countries. It has a highly versatile role in increasing economic yield in biofuel production, as animal feed, and as a raw material for many industries (Vathana et al. 2019). It is the third most important cereal crop, after rice and wheat (Erenstein et al. 2022). As a major cereal fodder crop, maize has superior characteristics, such as high palatability, freedom from anti-nutritional factors (Kifayat et al. 2022), 10.35% crude protein content, 22.99% acid detergent fibre (ADF), and 51.70% neutral detergent fibre (NDF) (Ali et al. 2015). Therefore, maize fodder is preferable for milk-producing animals and it is helpful for increasing body weight and milk production (Kifayat et al. 2022).

Utilization of heterosis for improving grain or fodder yield is primarily dependent on assessment of genetic diversity and identification of diverse inbred lines (Mukri et al. 2022). Parental selection from closely related genotypes leads to reduced variability and increased genetic depression and its predominantly relies on the morphological characterization of parents (Nyaligwa et al. 2015). Forage maize has significant variation in plant height, green fodder yield, and dry fodder yield (Pavithra et al. 2022) and also in nutritional traits, which are

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highly affected by environmental factors (Wang et al. 2016). So, the molecular markers are a valid option due to its free from environmental effects and facilitate assessment of diversity in a precise manner.

The various molecular markers include SSR markers, which have a very diverse role in plant breeding programmes. One of the major uses of these markers is a viable tool in unravelling the diversity of any gene pool at the genetic level (Rohini et al. 2020). SSR markers are considered ideal markers because they have tandem repeat lengths ranging from 2 to 6 base pairs, variable distribution across the genome, high levels of polymorphism, co-dominant expression, and reliable reproducibility (Kaur et al. 2015). The pairwise genetic dissimilarity estimation among the genotypes using SSR markers was effectively used for diversity analysis through clustering and Principal Coordinate Analysis (PCoA) in maize (Islam et al. 2023). Given the above, diversity analysis was carried out using 30 SSR markers to unravel the molecular genetic diversity among 28 fodder maize inbred lines. Furthermore, the association between the molecular genetic distance of 28 parents and their F_1 performance for various forage yield and quality traits was also determined.

MATERIAL AND METHODS

Twenty-eight fodder maize genotypes (Table 1) collected from the Department of Forage Crops, Tamil Nadu Agricultural University, Coimbatore, India, were used in this study. All inbreds were raised in separate pots and leaf samples were collected from 20-day-old seedlings from each genotype. A total of 30 SSR markers, which were evenly distributed throughout the genome, were selected at the rate of three per chromosome from the Maize Genetics and Genomics Database (MaizeGDB - https://www.maizegdb.org). The list of SSR markers used for this study and sequence information on them are presented in Supplementary Table 1.

Genotyping

Genomic DNA was isolated by following the cetyl trimethyl ammonium bromide (CTAB) extraction protocol (Murray and Thompson 1980). The pellet was suspended with 50 µL 1X TE buffer and 3 µL RNaseA and preserved at -20 °C. The DNA concentration (ng μL^{-1}) and quality were measured at 260 and 280 nm using Tecan's Infinite 200 NanoQuant. The

Table 1. List of genotypes used for SSR marker genotyping

Genotype	Pedigree	Source	S.No	Genotype	Pedigree	Source
UMI 61	Selection from Taiwan DMR 13	TNAU	15	DM 12-1	UMI 79/UMI 936 C1-100-12-1	TNAU
UMI 112	Selection from YUZP-SC-8	TNAU	16	DM 12-4	UMI 79/UMI 936 C1-67-12-4	TNAU
UMI 1200	Selection from W2619-3	TNAU	17	DM 12-5	UMI 79/UMI 936 C1-67-12-5	TNAU
UMI 1201	Selection from W2625-3	TNAU	18	DM 12-6	UMI 79/UMI 936 C1-29-12-6	TNAU
UMI 1205	Selection from W2620-2	TNAU	19	DM 74-2	UMI 79/UMI 936 C1-7-7-7-74-2	TNAU
N-09-160-2	S-123-160-2-1	TNAU	20	UMI 1210	Selection from W2619-3	TNAU
N-10-86	C 7250-86-4-5	TNAU	21	UMI 1221	Selection from TNAU N 148-1	TNAU
N 66	C 5180-66-10-7	TNAU	22	GETM 25	Derivatives from the UMI 1200/ UMI 112	TNAU
52021	Selection from CM-212	TNAU	23	GETM 26	Derivatives from the UMI 1201/ Co BC 1	TNAU
52485	Selection from W3206-2	TNAU	24	GETM 39	Derivatives from the UMI 71/Co 1	TNAU
DM 82	UMI 79/UMI 936-C1-7-7-7-82	TNAU	25	GETM 40	Derivatives from the UMI 1220/ UMI 76	TNAU
DM 84	UMI 79/UMI 936-C1-7-7-7-84	TNAU	26	GETM 67	Derivatives from the UMI 129/UMI 1210	TNAU
GETM 14	Derivatives from the UMI76/UMI 1220	TNAU	27	TNFM 139-1	Line derived from composite cross FDM 14,FDM 36, FDM 35, FDM 34, FDM 25, African Tall	TNAU
DM 12	UMI 79/UMI 936 C1-101-12	TNAU	28	African Tall	Line derived from composite cross H-611 C, H-611, H-611 (R)C3, K-III × EC-573 (R12) C3, Ukiri Comp A (F) C5 × Ukiri Comp A (F) C3, Chitedge Comp A and Ilonga Comp)	TNAU

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absorbance ratio of ~ 1.8 at these wavelengths indicates that the DNA is pure. The DNA was then re-suspended with MilliQ at the rate of 100 ng μL^{-1} DNA. Polymerase Chain Reaction (PCR) was performed for a 5 µL cocktail prepared with 0.5 µL template DNA, 2 µL Mastermix (2X), 2 µL MilliQ water, and 0.25µL each of forward primer and reverse primer (10 mM); and it was amplified using a thermal cycler (Applied Biosystems, USA; Veriti™ model)**.** In the PCR reaction, the following temperature profile was maintained: one cycle of initial denaturation at 94 ˚C for 7 minutes, 35 cycles of denaturation at 94 ˚C for 30 seconds, annealing for 30 seconds, and extension at 72 ˚C for 45 seconds, as well as one cycle of final extension at 72 ˚C for 7 minutes. The final products were separated by 3% agarose gel stained with ethidium bromide, along with a 100 bp ladder. The bands were visualized under the gel documentation system (Medicare, India; GELSTAN 4x Advanced model).

SSR data analysis

The bands were profiled by attributing 1 for the presence and 0 for the absence of bands for different alleles formed by a single marker for all genotypes. The polymorphic information content (PIC) was worked out using the following formula proposed by Botstein et al*.* (1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

where n =number of alleles, $p_i = i^{th}$ allele frequency, and $p_j = j^{th}$ allele frequency. The parameters of number of effective alleles (N_e), major allele frequency (MAF), Shannon's information index (I), heterozygosity (H), and the fixation index (F_{ST}) were worked out using the GenAlex 6.5 software (Peakall and Smouse 2005).

The scored binary data set is analysed using the DARWin 6.0.021 (Perrier et al. 2003). Based on total alleles produced by 30 markers, the corresponding genetic dissimilarity coefficients were analysed for 28 fodder maize genotypes using the Dice coefficient. The unweighted neighbour-joining dendrogram was constructed using the dissimilarity coefficients of all the genotypes and it was also used to view the cluster in graphical form using the pheatmap library in R Studio 4.2.1 (Kolde and Kolde 2015). The PCoA was performed in GenAlex 6.5 using the dissimilarity coefficient to understand the genetic differences among the genotypes.

Population structure analysis

The 28 inbred lines were analysed for population structure to determine the genetic structure. Using the STRUCTURE version 2.3.4 software (Pritchard et al. 2000), the possible number of subpopulations (K) was fixed based on the value with the maximum ΔK. Population structure was analysed with the following criteria: total burn-in period was 100,000 and number of Markov Chain Monte Carlo (MCMC) replications was also 100,000, along with the correlated admixture and allele frequency model. The number of subpopulations (K) was derived by using the range of 1 to 10, with five runs for each K. The true number of subpopulations was identified with the help of appropriate L (K) values by using the Evanno et al*.* (2005) method, extracted from the STRUCTURE HARVESTER software (Earl and VonHoldt 2012).

Assessment of genetic relationship between parents and hybrids

The genotypes which grouped in cluster analysis based on molecular diversity were used to develop single cross hybrids. A total of 195 hybrids were developed using 13 lines and 15 testers, which grouped in the first and second major clusters. Hybrid performance was evaluated over three seasons: rainy 2022, winter 2022, and summer 2023. A randomized block design was adopted to evaluate the performance of the hybrids, with two replications. Two rows at a spacing of 30 cm × 15 cm were grown for each hybrid. The observations recorded for sixteen traits included quality parameters such as days to fifty per cent flowering, plant height, cob placement height, leaf length, leaf breadth, number of leaves, number of nodes, internode length, stem girth, leaf stem ratio, green fodder and dry matter yields, crude protein (CP), crude fiber (CF), acid detergent fiber (ADF), and neutral detergent fiber (NDF). The forage quality traits (CP, CF, ADF, and NDF) were analysed using a Near Infrared Spectrophotometer (NIR), SpectraAlyzer ZEUTECH model.

RESULTS AND DISCUSSION

SSR markers were used in this study to determine the genetic dissimilarity among the 28 fodder maize genotypes.

The results revealed that all markers registered polymorphism among the genotypes, with a total of 110 alleles (Table 2) for 30 SSR markers – 2-7 alleles per locus, an average of 3.667.

The banding profile of the 28 fodder maize inbreds generated by the SSR markers *bnlg128* and *umc1538* are presented in Figure 1a,b. The *bnlg128* marker produced seven polymorphic alleles and *umc1538* produced six, among the 28 fodder maize inbreds, which revealed that high genetic divergence prevailed among the genotypes studied. Mathiang et al. (2022) reported an average of 7.4 alleles per loci in screening 37 maize landraces using 27 SSR markers. None of the markers was found to be monomorphic in all the genotypes screened. A minimum of two alleles were scored for eight markers and a maximum of 7 alleles for *mmc0351* and *bnlg128* (Table 2). The number of effective alleles (Ne) assesses the presence of equally frequent alleles per locus over the given population. It ranged from 1.036 (*phi035*) to 4.558 (*bnlg128*), with an average of 2.604. The major allele frequency ranged from 0.286 (*umc1538*) to 0.982 (*phi035*), with an average of 0.544. Shannon's information index (I) ranged from 0.090 (*phi035*) to 1.648 (*bnlg128*), with an average of 0.996. The fixation index (F) is the coefficient that measures inbreeding, indicating how the genotypic proportions in the population deviate from Hardy-Weinberg equilibrium for a specific locus (Suvi et al. 2020). In this study, the F value was observed as zero for the markers *umc1625* and *phi035* and maximum of one in eight markers (*mmc0151*, *mmc0081*, *umc2538*, *umc1408*, *umc1530*, *umc2616*, *umc1867*, and *phi062*), with an average of 0.659. This indicates that appreciable level of homozygosity in the studied inbreds.

Table 2. Diversity characteristics of SSR markers in 28 fodder maize inbreds

Locus	Na	Ne	MAF	I	H	F_{ST}	PIC
umc1566	3	1.931	0.679	0.833	0.482	0.556	0.501
umc1035	6	4.126	0.375	1.549	0.758	0.859	0.721
umc1538	6	4.094	0.286	1.533	0.756	0.480	0.745
umc1028	4	3.496	0.407	1.320	0.714	0.896	0.676
umc2246	3	1.919	0.661	0.789	0.479	0.925	0.419
umc2184	3	1.840	0.679	0.740	0.457	0.844	0.383
bnlg1447	5	4.062	0.304	1.476	0.754	0.810	0.710
bnlg2241	4	2.365	0.536	0.990	0.577	0.814	0.543
bnlg1182	4	2.326	0.589	1.037	0.570	0.499	0.552
umc1757	5	4.442	0.321	1.549	0.775	0.170	0.711
bnlg490	5	2.405	0.554	1.078	0.584	0.755	0.549
umc1854	4	2.788	0.500	1.171	0.641	0.076	0.625
mmc0151	5	3.267	0.464	1.352	0.694	1.000	0.649
mmc0351	7	3.867	0.389	1.545	0.741	0.201	0.726
mmc0081	3	1.894	0.679	0.798	0.472	1.000	0.409
umc1625	5	3.853	0.357	1.437	0.740	0.000	0.672
umc1352	2	1.849	0.643	0.652	0.459	0.689	0.353
umc2538	2	1.960	0.571	0.683	0.490	1.000	0.369
umc1408	2	1.415	0.821	0.469	0.293	1.000	0.250
phi057	3	2.575	0.446	1.003	0.612	0.533	0.557
umc2197	3	2.166	0.607	0.900	0.538	0.602	0.504
umc1530	2	2.000	0.500	0.693	0.500	1.000	0.375
umc1638	2	1.997	0.518	0.693	0.499	0.785	0.372
umc2616	2	1.912	0.607	0.670	0.477	1.000	0.363
bnlg128	7	4.558	0.321	1.648	0.781	0.497	0.736
umc1867	3	1.668	0.750	0.712	0.401	1.000	0.358
bnlg1159	3	2.469	0.536	0.990	0.595	0.640	0.549
phi041	3	1.856	0.696	0.798	0.461	0.303	0.447
phi062	2	1.990	0.536	0.691	0.497	1.000	0.373
phi035	2	1.036	0.982	0.090	0.035	0.000	0.064
Mean	3.667	2.604	0.544	0.996	0.561	0.664	0.509

Na = no. of different alleles, Ne = no. of effective alleles, MAF = major allele frequency, I = Shannon's information index, H = heterozygosity, F_{ST} = fixation index, PIC = polymorphic information content

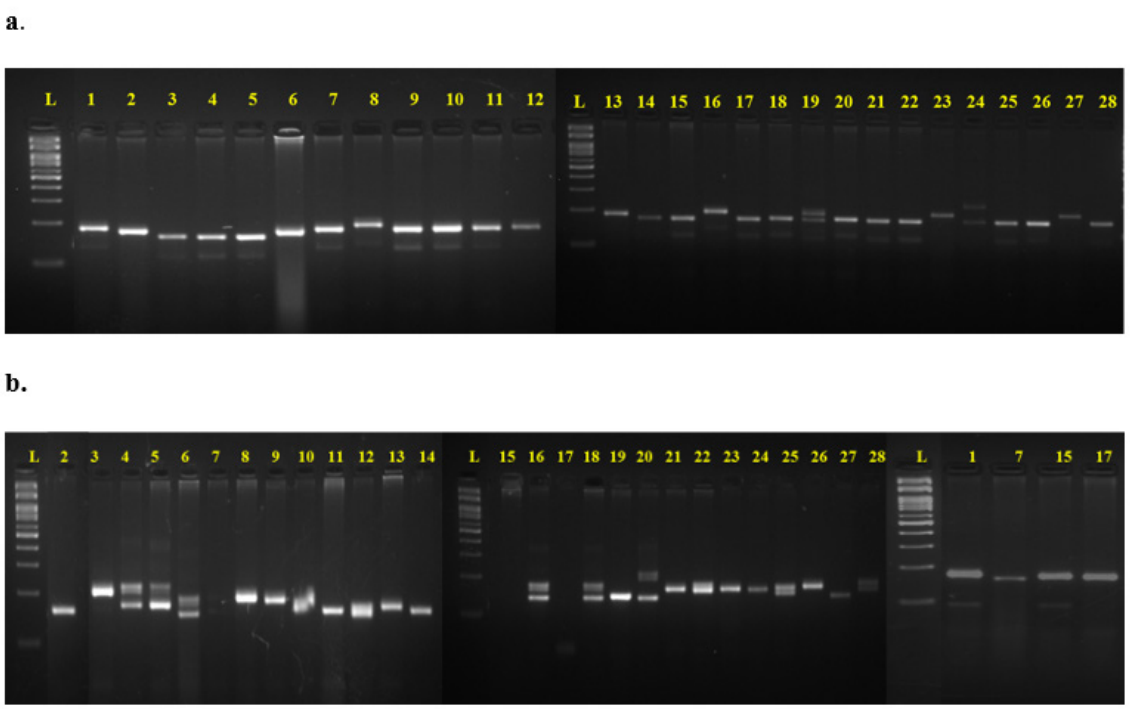

Figure 1. **a**. DNA profile of 28 fodder maize inbreds with SSR marker bnlg128; **b**. DNA profile of 28 fodder maize inbreds with SSR marker umc1538.

In quantitative terms, the degree of genetic polymorphism was measured by two major parameters: H and PIC. Among the 30 markers (Table 2), a maximum H value was observed in the marker *bnlg128* (0.781), followed by *umc1035* (0.758). The marker *umc1538* recorded the highest PIC value, 0.745, with 6 alleles. It was followed by *bnlg128* with a PIC value of 0.736. The marker *phi035* recorded very low values for both H and PIC content (0.035 and 0.064, respectively). The average content of H was 0.561 and 0.509 for PIC. The high H value implies that the genotypes under study exhibited greater genotypic variability for the adaptive traits (Adu et al. 2019). These results were similar to the study by Bernard et al. (2018). African Tall is a composite population, which may have contributed to a higher level of heterozygosity for some markers. The H value may be defined as the level of probability of individual heterozygosity for a locus in the population under study (Botstein et al. 1980). In the case of PIC, it is a discriminatory capability of a marker to develop polymorphism over the population. The marker with > 0.5 PIC is stated as more informative than one with < 0.25 PIC (Botstein et al. 1980). In this current evaluation, about 60.71 per cent of markers were identified as more informative (PIC > 0.5). Comparing the PIC and H with number of alleles per locus, results indicated that allele richness is positively correlated with increased PIC (r = 0.86) and H (r = 0.78). These results indicate the huge allelic variations for most of the SSR markers used in the population studied, as observed by Adu et al. (2019).

Population structure analysis

The population structure among 28 fodder maize inbred lines determined by structure analysis revealed the highest peak at K=4 (Supplementary Figure 1). Based on K=4, the genotypes in this study were divided into four groups, with the ΔK value of 132.70. The inferred ancestry values of the genotypes is given in Supplementary Table 2. Group three (G3) had the highest number, nine individuals, followed by group two (G2), which had seven genotypes. Group one (G1) and group four (G4) had six genotypes each (Figure 2). Divergence in allele frequency was highest (0.226) between G2 and G4, while the lowest divergence (0.095) was observed between G3 and G4 (Table 3). The expected heterozygosity among the individuals within the same group was highest in G3 (0.529) and lowest in G2 (0.382). The F_{ST} values were used to reveal genetic variation among the subgroups. The highest F_{ST} value (0.410) of G2 indicated that this population was

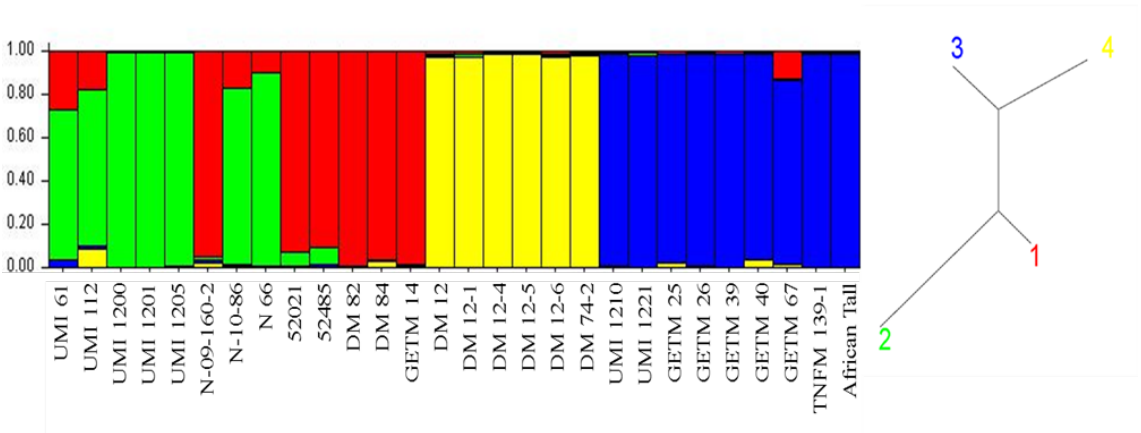

Figure 2. Population structure analysis of 28 fodder maize genotypes.

Table 3. Allele-frequency divergence and heterozygosity values of subpopulations

Allele-frequency divergence among populations (net nucleotide distance)					Average distances (expected heterozygosity) between individuals in same cluster		
Group	**G1**	**G2**	**G3**	**G4**	**Heterozygosity**	**F_{ST}**	**No. of genotypes**
G1	-				0.511	0.222	6
G2	0.1227	-			0.382	0.410	7
G3	0.1342	0.1801	-		0.529	0.159	9
G4	0.1332	0.2263	0.0955	-	0.434	0.323	6

most structured. Similar reports of genetic diversity and population structure analysis were made by Choudhary et al. (2023) in fodder maize landraces.

Diversity analysis

Based on the dissimilarity coefficient, 28 fodder maize genotypes were grouped into two major clusters and four sub-clusters (Figure 3). This grouping pattern was very similar to population structure analysis. Cluster I had eleven genotypes and Cluster IV had nine genotypes, followed by Cluster III with six and Cluster II with two. This indicated the distinctiveness of genotypes for the 30 SSR markers studied. The pairwise Dice dissimilarity genetic distance of 28 genotypes is shown in Supplementary Figure 2. The genetic distance of inbred lines ranged from 0.063 to 0.777, with an average of 0.534. The smallest genetic distance (0.063) was observed between the genotypes UMI 1201 and UMI 1205, whereas the highest dissimilarity (0.777) was observed between DM 84 and UMI 1221. Genotypes with higher dissimilarity would be useful for synthesizing heterotic single cross fodder maize hybrids or for developing diverse inbreds through selection in segregating progenies. Nyaligwa et al. (2015) clustered 79 elite lines of maize inbreds into three major clusters and a few sub-clusters using 30 SSR markers.

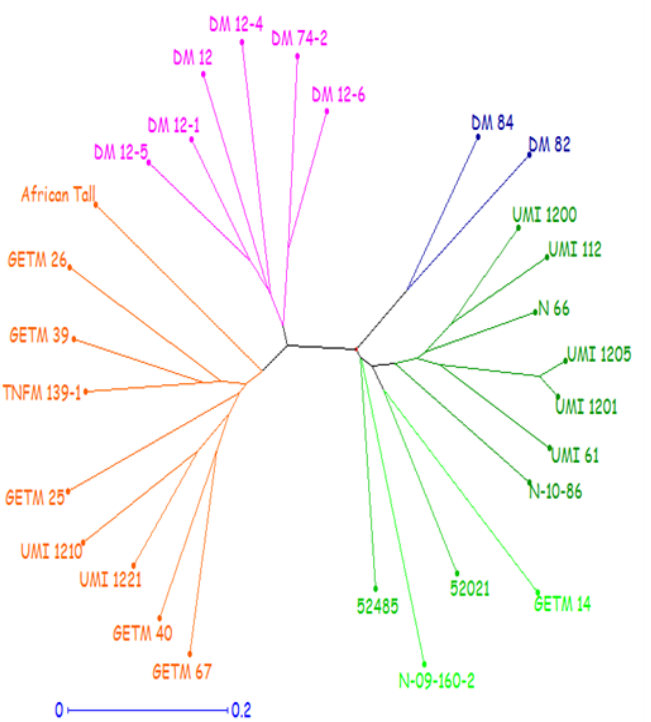

Figure 3. Relationship among the 28 fodder maize inbreds detected by 30 SSR markers.

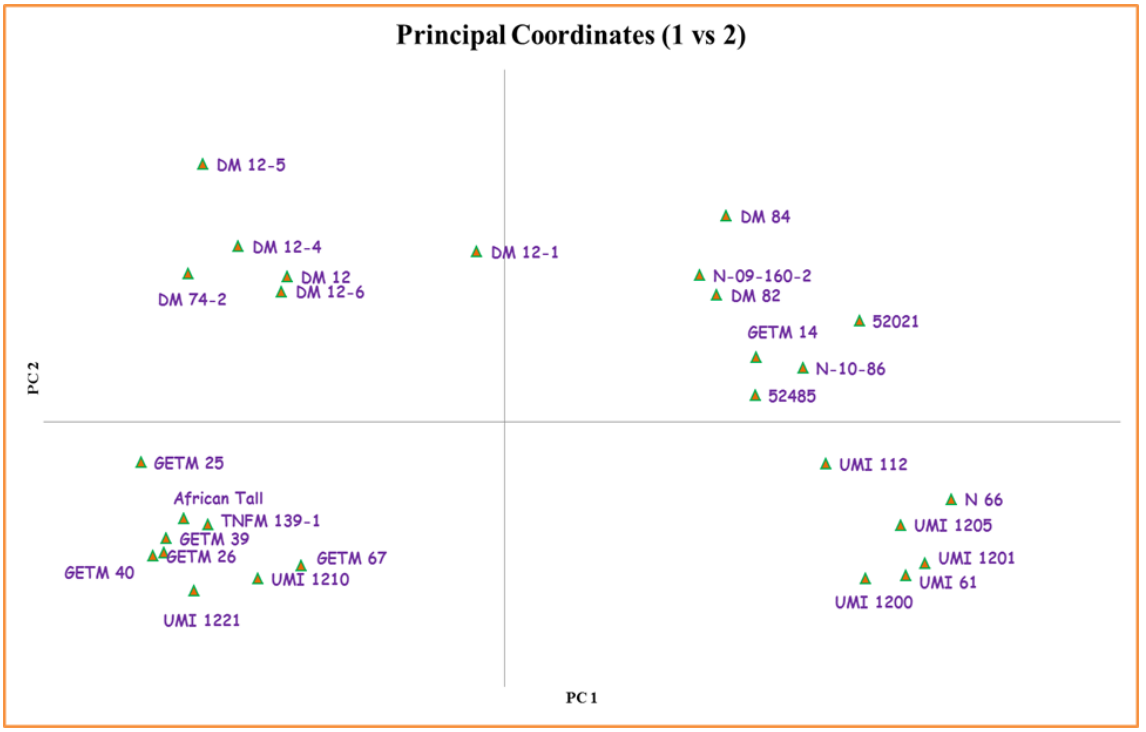

Figure 4. Principal Coordinate Analysis (PCoA) based on 30 SSR markers.

To know the spatial distribution of fodder maize genotypes, PCoA was also carried out. The graphical depiction of PCoA is shown in Figure 4. The first two principal coordinates (PCs) accounted for 29.62% of the total variance, with the first PC contributing 18.56% and the second PC contributing 11.06%. Based on the SSR marker survey, the genotypes were separated into four heterotic groups, and the grouping pattern in both the clustering approach and the PCoA were quite similar. Oliveira et al. (2021) gathered 293 maize inbreds into four groups by this approach.

Association between molecular genetic distance of 28 parents and their 195 hybrids

Correlation was analysed between pairwise genetic distances determined from the molecular diversity of parents and the mean performance of the resulting hybrids (Table 4). The results indicated significant positive association for cob placement height and crude protein content in the first season (rainy 2022). But neither trait had significant correlation in the second (winter 2022) and third (summer 2023) seasons with the parents genetic distance. In the second season, number of leaves, number of nodes, and leaf stem ratio had a significant relationship with their parental geneti distance. In the third season, leaf stem ratio

Table 4. Association between genetic distance of 28 parents and their 195 hybrids

Trait	rainy 2022	winter 2022	summer 2023
DFF	-0.07	-0.108	-0.031
CH	**0.16***	0.134	0.014
PH	0.07	0.086	-0.015
NL	0.02	**0.157***	0.023
NN	-0.01	**0.145***	0.023
LL	0.01	-0.004	0.000
LB	-0.01	-0.046	0.016
IL	0.01	0.037	-0.051
SG	0.05	0.045	-0.063
LSR	-0.06	**-0.151***	**-0.206***
CP	**0.15***	0.05	-0.067
CF	-0.07	-0.059	-0.062
ADF	-0.04	-0.077	-0.044
NDF	-0.08	-0.101	-0.042
DMY	-0.13	0.049	0.132
GFY	-0.09	0.038	0.074

*significance level @ 5%, DFF – days to fifty per cent flowering, CH – cob placement height, PH – plant height, NL – number of leaves, NN – number of nodes, LL – leaf length, LB – leaf breadth, IL – internode length, SG – stem girth, LSR – leaf stem ratio, CP – crude protein, CF – crude fibre, ADF – acid detergent fibre, NDF – neutral detergent fibre, DMY – dry matter yield, GFY – green fodder yield.

had a negative association. Among these traits, leaf stem ratio consistently had a significant negative association in both seasons. Geng et al. (2021) also observed a significant association between molecular genetic distance of parents due to SSR markers and their F_1 performance for yield-related traits. The inconsistency of association between molecular diversity and hybrid performance may be due to the G × E interaction and insufficient marker coverage of the whole genome. Hence, more markers like SNPs with whole genome coverage may help to ascertain the relationship between parental molecular diversity and hybrid performance.

According to polygenic trait inheritance, superior heterotic performance is the result of parental divergence. But whether we need to select parents with wider or narrower diversity of superior hybrid performance is always a dilemma. Many studies on morphological diversity have indicated that a medium level of parental diversity is helpful to obtain good heterotic hybrids. However, the effect of parental diversity on various traits, such as quality and yield components, needs to be considered, especially in a forage breeding programme. Based on the results, the molecular diversity of the parental lines was not able to assist in arriving at any conclusion on hybrid performance. However, the morphological diversity of the parents helped predict the performance of hybrids for days to 50% flowering and crude protein. Both these traits are highly important in a forage breeding programme. We can conclude that an average or small level of parental diversity may be useful to obtain hybrids with medium/early flowering and moderate/high crude protein content.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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