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GENETIC AND MOLECULAR DIVERSITY ANALYSIS IN FORAGE SORGHUM (*SORGHUM BICOLOR* (L.) MOENCH)

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SUMMARY

The present investigation was carried out to find the diverse genotypes by using D^2 statistics and SSR markers. The field-level experiment was laid out in randomized block design with thirty-five genotypes during *Kharif*-2021 at Centre for Millets Research, Deesa. The genotype where grouped into thirteen clusters based on Mahalanobis D^2-statistics. The maximum inter-cluster distance was observed between cluster XII and XIII (27.19), thus genotypes included in these clusters may be utilized under an inter-varietal hybridization programme for forage yield improvement. In case of molecular diversity analysis, the number of al-leles for SSR primers ranged from two to four with an average number of alleles per locus was 2.73. The similarity coefficients among all the 35 genotypes ranged from 0.05 to 1.00. The clustering pattern of dendrogram generated by pooled molecular data of seventeen SSR loci generated two clusters *viz.,* A and B at a similarity coefficient of 0.56 and cluster A is subdivided into sub clusters A_1 ($A_{1:1}$ and $A_{1:2}$) and A_2 ($A_{2:1}$ and $A_{2:2}$), while cluster B subdivided into sub cluster B_1 and B_2. The combined results for morphological and molecular diversity (using SSR markers) estimates that genotype "Gundari" was distinct from other genotypes and can be exploited to harness their unique features in breeding programmes.

Key words: Forage sorghum, genetic diversity, molecular diversity

Sorghum [*Sorghum bicolor* (L.) Moench] is the most important cereal crop grown in the semi-arid tropics of Africca, Asia, Australia, and America (Tonapi *et al.,* 2020). It belongs to *Poaceae* family and tribe Andropogoneae (Harlan and de Wet, 1972). It is also known as great millet, Indian millet, milo, durra and orshallu. Anthropological evidences suggest that hunters and gatherers used to consume sorghum as early as 8000 BC (Smith and Fredriksen, 2000). Sorghum is consumed in different forms for various end-uses. Its grain is mostly used directly for food purposes. Sorghum is a good fodder crop for creating hay and silage. In comparison to grain sorghum, forage sorghum plants grow 6 to 12 feet tall and yield higher dry matter content. The stalks and leaves are covered with white wax, and some varieties have sweet, juicy pith in the middle of the stalks. It posses 2n=20 chromosome number and the genome size 730Mb, which makes sorghum an attractive model for functional genomics of *Saccharinae* and other C_4 grasses.

In India, concerted breeding efforts for the improvement of forage sorghum were initiated in 1970 under the All India Coordinated Research Program on Sorghum and subsequently many improved single-cut and multicut varieties and hybrids were developed.

In order to make forage sorghum as an enterprising and remunerative crop, there's necessary to improve varieties or hybrids with desired characters such as faster growth, good vigour and high forage yield, high protein content, and low HCN content at the flowering stage for the fodder quality and utilization. To develop such forage varieties or hybrids, knowledge and information on genetic architecture is essential for the formulation of an efficient breeding strategy for genetic improvement of sorghum as forage crop. The genetic diversity of plants determines their potential for improved efficiency and hence their use

for breeding, may eventually result in enhanced food production. The best way to understand the potential of the available germplasm is by analyzing its genetic diversity. For an outstanding breeding programme in the crop improvement, diversity analysis greatly helps the breeder in the identification and proper choice of parents for specific breeding objectives. Selection of genetically diverse parents is expected to yield higher frequency of heterotic hybrids in addition to generating a broad spectrum of variability in segregating populations. Mahalanobis D^2 statistics based on multivariate analysis of quantitative traits is a powerful tool for measuring divergence in a set of population using the concept of statistical distances utilizing multivariate measurement (Mahalanobis, 1936).

Molecular markers play a major role in selecting diverse genotypes. Microsatellites are important genetic markers in identification and characterization of plant species. These are co-dominant and PCR-based. Microsatellites are highly polymorphic and evenly spread throughout a genome (Areshchenkova and Ganal, 1999). These are found to be highly reproducible and cost effective (Jones *et al.*, 1997). Simple sequence repeats (SSRs) for variety profiling can provide high discrimination and excellent reproducibility.

MATERIALS AND METHODS

Genetic diversity analysis

The experimental material comprising of thirty-five forage sorghum genotypes were evaluated in randomized block design with three replications at the Centre for Millets Research, Sardarkrushinagar Dantiwada Agricultural University, Deesa during *Kharif* – 2021. Each genotype was sown in one row of two-metre length with optimum inter-row spacing of 30 cm. The observations were recorded both as visual assessment for days to 50 per cent flowering and measurement on five randomly selected plants for plant height (cm), number of leaf per plant, stem girth (mm), leaf length of blade (cm), leaf width of blade (cm), leaf: stem ratio, brix content (%), hydrocyanic acid content (ppm), crude protein content (%), green fodder yield per plant (g) and dry fodder yield per plant. For the statistical analysis of all the traits under study, mean values of different traits were taken from five randomly chosen plants from each genotype, and the mean value of the plot in each replication. The genetic diversity analysis was carried out by D^2 statistics as proposed by Mahalanobis (1936) and described by Rao (1952). Grouping of genotypes into different clusters was done by Tocher's method. The intra-cluster and inter-cluster distances were calculated by the formula given by Singh and Chaudhary (1977).

Molecular diversity analysis

The molecular analysis was carried out at the Department of Genetics and Plant Breeding, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. Healthy fresh leaves were collected from all the genotypes at the seedling stage for DNA extraction. High molecular weight genomic DNA was isolated from 35 sorghum genotypes by following CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method as described by Doyle and Doyle (1987) with some modifications. The list of primers selected for the study is shown in Appendix A. Out of which thirteen SSR primers were selected and used after their optimization and screening.

Scoring and statistical analysis of data

The amplified products of each primer were scored separately based on the presence or absence of a band among 35 sorghum genotypes *i.e.,* use of binary codes 1 and 0 for the presence or absence of a band, respectively. The data were entered into a binary matrix and subsequently analyzed using NTSYSpc version 2.20 software package (Rohlf, 2000).

The similarity matrix coefficient among the 35 sorghum genotypes were estimated by using Jaccard's similarity co-efficient (Jaccard, 1908) and cluster analysis was done by using the Un-weighted pair group method and arithmetic average (UPGMA) function of NTSYSpc (version 2.20). The diversity parameters such as heterozygosity and polymorphism information content were calculated using formulas given by Binkowski and Miks (2018). The software program Alpha Ease FC version 4.0.0 developed by Alpha Innotech Corporation, U.S.A. was used for determining the Molecular Weight (MW) of bands separated on the gel. Heterozygosity (H) is a parameter indicating the average frequency of heterozygous individual's occurrence. Polymorphic information content (PIC) is a measure of locus polymorphism counted for given DNA markers used in linkage analysis for the 35 forage sorghum genotypes. The marker data were then standardized for Principle

coordinate analysis (PCoA) using DARwin (version 6.0) (Perrier and Jacquemound, 2006).

RESULTS AND DISCUSSION

Genetic diversity analysis

For improving complex trait like forage yield, the selection of parents having wide divergence for various characters is of prime importance which can be assessed by D^2 statistics developed by Mahalanobis (1936). The greater distance between two clusters indicates greater divergence and vice versa. The genotypes falling in the same cluster indicates more closely related than those belonging to other clusters. In view of this, Mahalanobis D^2 statistic was used to assess the genetic divergence in different forage sorghum genotypes to identify superior genotypes which can be utilized for the future breeding programmes.

With help of D^2 values, thirteen clusters were generated from 35 genotypes with 12 traits of forage sorghum. Table 1 shows the composition of clusters.

The results indicated that a maximum number of fourteen genotypes appeared in cluster I followed by six genotypes in cluster IV, four genotypes in cluster VI, two genotypes in cluster XII and each one genotype in clusters II, III, V, VII, VIII, IX, X, XI and XIII. This indicated that there is a presence of diversity among the 35 genotypes studied. The genotypes IS-3222, SRF-420, GAFS 12, DSF-219, DS-1077, DSF-224, Gundari and Gundlopet were found each in distinct cluster revealed that there may presence of distinguished trait compared to other studied genotypes. The average intra and inter-cluster distance between all possible pairs of thirteen clusters are presented in Table 2 and Fig. 3.

Inter-cluster D^2 values ranged from 9.25 to 27.19. The maximum inter-cluster distance was observed between cluster XII and XIII (27.19) and minimum inter-cluster distance was observed between cluster II and IV (9.25). The intra-cluster distance ranged was from 0.0 to 10.76. The maximum intra-cluster distance 10.76 followed by 10.65, 10.37 and 10.32 were observed in cluster VI, I, XII and IV respectively. While, the least intra-cluster distance (*i.e.*, 0.0) was observed in rest of clusters *viz.*, II, III, V, VII, VIII, IX, X, XI and XIII as they have single genotype. The inter-cluster distances in the current analysis were greater than the intra-cluster distances, indicating greater genetic diversity across the various accessions of different clusters than those of the same cluster. Similar results were reported by Elangovan *et al.* (2014), Meena *et al.* (2016), Ahalawat *et al.* (2018) and Arvinth (2021). The maximum inter-cluster distance was observed between cluster XII and XIII indicated that, the genotypes included in these clusters are more diverse and may generate high heterotic response in breeding program. Similar results were reported earlier by Tesfaye (2017) and Pal *et al.* (2022).

The contribution of different traits towards divergence was shown in Table 3. Brix content (26.72%) was the main contributor to the total genetic divergence followed by days to flowering (23.53%) and HCN content (18.82%). In contrast, the character

TABLE 1
Distribution of 35 genotypes of forage sorghum in different clusters on the basis of D^2 statistics

Cluster	Total number of genotypes	Name of genotype
I	14	SH-1488, S-652, SRF-421, SRF-422, DSF-226, SSG 59-3, DS-1158, DS-1059, MALWAN, SH-1813, GFS 6, DS-1156, SRF-423 and CSV 46F
II	1	IS-3222
III	1	SRF-420
IV	6	PJDP-1612/621513, DS-1188, GFS 4, SOR-8586, AFS-68 and PJDP-1619/621520
V	1	GAFS 12
VI	4	CSV 33MF, CSV 41, CSV 27 and DSF-222
VII	1	DSF-219
VIII	1	Makari Jowar
IX	1	DS-1077
X	1	DSF-224
XI	1	Gundari
XII	2	IC-4852 and SRF-407
XIII	1	GUNDLOPET

TABLE 2
Average Intra-cluster (Diagonal) and Inter-cluster (off diagonal) distance of 13 clusters of forage sorghum

Cluster	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
I	10.65	12.52	13.50	13.06	13.81	14.73	12.81	14.93	13.32	15.87	16.49	18.26	15.83
II		0.00	17.96	9.25	17.21	19.06	14.71	19.40	13.10	17.44	19.88	18.46	21.41
III			0.00	16.23	11.78	11.08	15.04	10.35	17.91	11.34	18.17	12.67	17.65
IV				10.32	16.26	18.81	16.47	10.09	15.64	15.97	21.74	16.50	22.16
V					0.00	13.02	20.72	15.71	17.76	18.58	21.85	19.90	15.25
VI						10.76	17.31	15.10	18.98	18.24	19.12	17.18	17.74
VII							0.00	13.51	12.44	13.97	9.88	18.84	17.49
VIII								0.00	14.66	11.94	12.24	19.03	13.50
IX									0.00	14.76	11.38	22.13	15.14
X										0.00	16.92	13.47	21.08
XI											0.00	24.51	13.47
XII												10.37	27.19
XIII													0.00

TABLE 3
Per cent contribution of different characters towards total genetic divergence of forage sorghum

S. No.	Characters	Time ranked 1st	Contribution to divergence (%)
1.	Days to flowering	140	23.53
2.	Plant height	4	0.67
3.	Number of leaf per plant	3	0.50
4.	Stem diameter	1	0.17
5.	Leaf length of blade	0	0
6.	Leaf width of blade	1	0.17
7.	Leaf: stem ratio	12	2.02
8.	Brix content	159	26.72
9.	HCN content	112	18.82
10.	Crude protein content	68	11.43
11.	Green fodder yield per plant	58	9.75
12.	Dry fodder yield	37	6.22

leaf length of blade showed no contribution toward total genetic divergence.

Cluster means for different characters

The mean performance of cluster values for twelve characters is presented in Table 5. The cluster mean values observed among different characters *viz.,* days to flowering. plant height (cm), number of leaf per plant, stem diameter (mm), leaf length of blade (cm), leaf width of blade (cm), leaf: stem ratio, brix (%), HCN content (ppm), crude protein content (%), green fodder yield per plant (g) and dry fodder yield per plant (g). In the present study, a wide range of cluster mean was observed for various characters in forage sorghum. Cluster VIII exerted maximum mean values for important characters *viz.,* number of leaf per plant (12.80), stem diameter (15.53 mm) and crude protein content (11.54 %), cluster XI for leaf width of blade (8.11 cm) and green fodder yield per plant (478.02 g), cluster XII for leaf: stem ratio (0.27) and HCN content (75.02 ppm), XIII for days to flowering (79.00 days) and dry fodder yield (140.06 g), cluster II for plant height (293.67 cm), cluster X for leaf length of blade (90.19 cm) and cluster VII for brix content (13.73%). The minimum values were depicted for stem diameter, days to flowering, brix content and HCN content in cluster V (8.74), cluster X and XII (57.67%), cluster V (8.05 ppm) and cluster V (3.91), respectively. Therefore, the genotypes present in these clusters may be used for improving the specific character for forage yield improvement.

MOLECULAR DIVERSITY ANALYSIS

SSR marker analysis of 13 SSR marker pairs *viz.*, msbCIR240, msbCIR300, msbCIR329, Xcup14, Xcup53, Xtxp12, Xtxp15, Xtxp67, Xtxp141, Xtxp265 and Xtxp317 generated a total of 32 alleles among 35 forage sorghum genotypes. Number of alleles ranged from two to four with an average number of 2.73 alleles per locus. This result was closely related with the findings of Agrama and Tuinstra (2003), Rakshit *et al.* (2012), Joshi *et al.* (2020) and Arvinth (2021). The maximum number of four alleles were recorded in msbCIR240, followed by three alleles were recorded in Xcup53, Xtxp12, Xtxp15, Xtxp67, Xtxp141, and Xtxp265, and a minimum number of two alleles in msbCIR300, msbCIR329, Xcup14 and Xtxp317. The primers Xtxp283 and Xtxp358 were found monomorphic. Arvinth (2021) also reported that the primer Xtxp358 was monomorphic.

TABLE 4
Results of SSR analysis in thirty-five genotypes of forage sorghum

S. No.	SSR markers	Molecular band size (bp)	Number of alleles	PIC	Heterozygosity
1.	msbCIR240F	108-170	4	0.56	0.00
2.	msbCIR300F	106-119	2	0.19	0.06
3.	msbCIR329F	114-133	2	0.40	0.00
4.	Xcup14F	206-256	2	0.13	0.03
5.	Xcup53R	180-203	3	0.50	0.03
6.	Xtxp12R	158-226	3	0.54	0.16
7.	Xtxp15F	200-225	3	0.49	0.09
8.	Xtxp67F	164-206	3	0.74	0.12
9.	Xtxp141F	118-169	3	0.64	0.16
10.	Xtxp265F	168-208	3	0.26	0.13
11.	Xtxp283F	254	1	0.00	0.00
12.	Xtxp317F	150-168	2	0.53	0.03
13.	Xtxp358F	209	1	0.00	0.00
	Average		2.73	0.38	0.06

TABLE 5
Cluster mean for twelve different characters in thirty-five genotypes of forage sorghum

Cluster	DF	PH	NL	SD	LL	LW	LS	BR	HCN	CP	GFY	DFY
I	69.21	230.99	8.51	10.99	70.90	6.01	0.20	11.70	30.66	9.45	229.54	84.82
II	60.67	293.67	10.77	10.86	65.59	5.43	0.18	13.40	25.00	7.64	204.19	62.16
III	67.67	254.40	7.10	10.79	81.83	4.98	0.18	8.82	54.19	11.40	228.53	65.77
IV	58.50	227.52	9.32	9.06	63.11	5.64	0.20	12.23	23.77	9.58	166.98	61.18
V	67.33	266.00	7.37	8.74	68.01	4.68	0.14	8.05	3.91	9.45	212.05	64.47
VI	74.50	256.15	8.23	10.31	67.94	5.49	0.18	8.32	56.82	8.21	196.00	60.69
VII	72.33	256.73	10.40	11.94	85.37	6.81	0.22	13.73	68.55	10.06	311.07	108.06
VIII	73.00	260.87	12.80	15.53	82.93	7.28	0.18	9.69	44.57	11.54	396.47	96.88
IX	66.00	238.93	10.40	11.50	71.83	6.71	0.20	12.40	29.99	7.34	413.56	127.78
X	57.67	247.00	8.33	12.79	90.19	7.45	0.19	10.46	55.83	11.19	278.30	107.40
XI	77.00	276.80	10.87	13.86	68.26	8.11	0.17	12.66	57.17	9.46	478.02	132.56
XII	57.67	235.97	7.85	9.77	72.78	5.35	0.27	9.24	75.02	10.15	110.87	44.15
XIII	79.00	268.13	10.30	14.66	58.06	6.19	0.13	10.99	8.03	10.58	400.35	140.06
Mean	67.73	254.86	9.40	11.60	72.83	6.16	0.19	10.90	41.04	9.70	278.92	88.92

Where, DF - Days to flowering (days), PH - Plant height (cm), LN - Number of leaf per plant , SD - Stem diameter (mm), LL - Leaf length of blade (cm), LW - Leaf width of blade (cm), LS - Leaf: stem ratio, BR - Brix content (%), HCN - Hydrocyanic acid content (ppm), CP - Crude protein content (%), GFY - Green fodder yield per plant (g), DFY - Dry fodder yield per plant (g).

The genotypes were subjected to Jaccard's coefficient analysis for finding the similarity between all possible pairs of genotypes. The results indicated that the similarity coefficients among all the 35 genotypes ranged between 0.05 to 1.00. The genotype Gundari found most diverse with the genotypes AFS-68 and IS-3222, also genotype DSF-19 found most diverse with the genotype IS-3222 with similarity coefficient 0.05. Whereas genotype DS-1077 found similar to genotype DS-1059 with similarity coefficient 1.00 as compared to other genotypes.

The molecular size of the amplified PCR products ranges from 106bp (msbCIR300) to 256bp (Xcup14). The PIC value ranged from 0.13 (Xcup14) to 0.74 (Xtxp67) with an average of 0.38.

To study the phylogenetic/evolutionary relationship among different genotypes, the dendrogram was constructed by using 13 SSR markers through NTSYSpc (version 2.20) on software (Fig. 2). Based on dendrogram, two main clusters were formed from 35 genotypes *i.e.*, cluster A and cluster B which were formed at similarity coefficient of 0.56. Cluster A is subdivided into two sub clusters *viz.*, A_1 and A_2. The sub cluster A_1 includes Cluster $A_{1:1}$

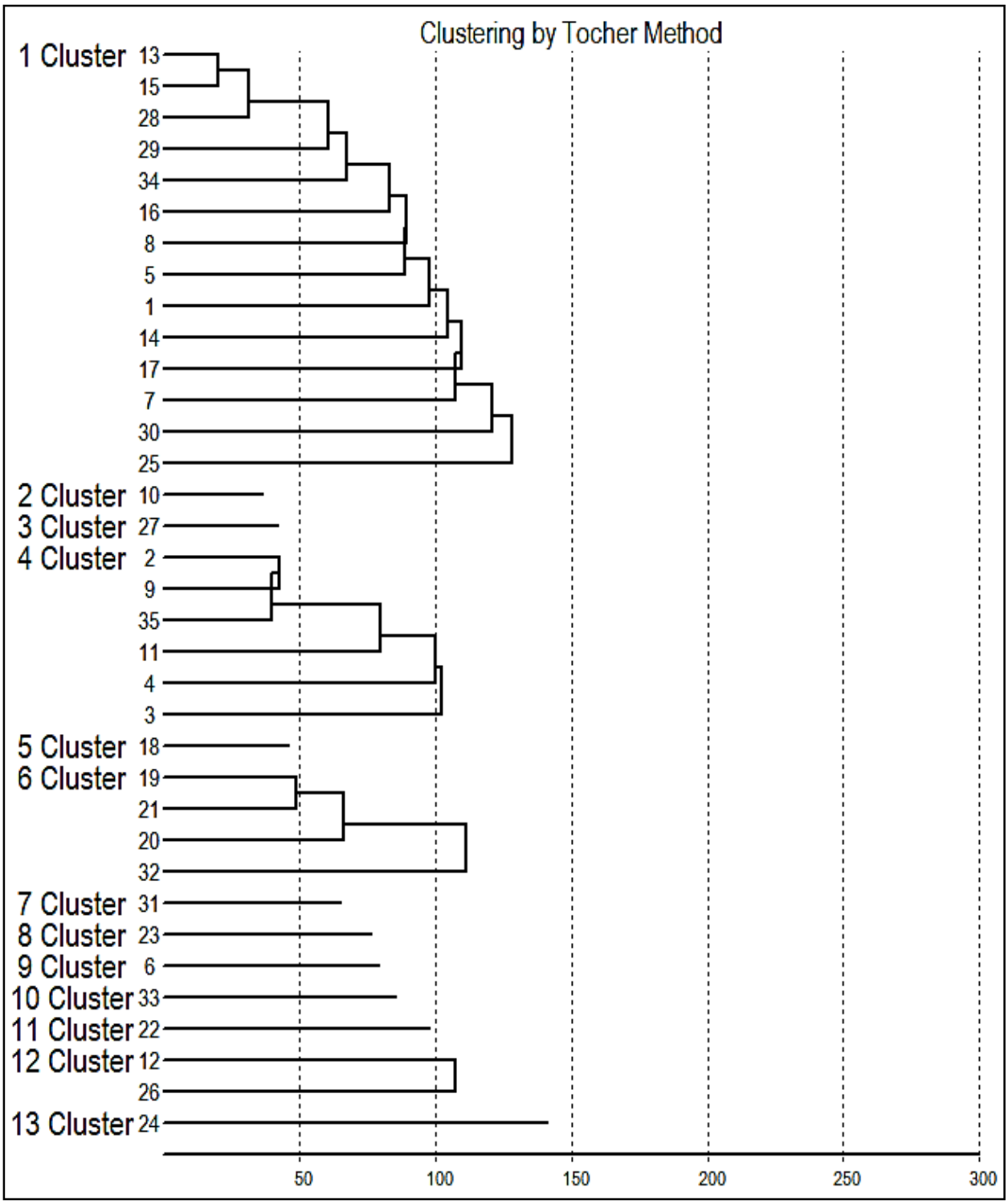

Fig. 1. Cluster diagram showing interrelationship among eight clusters using D^2 analysis.

1. MALWAN
2. PJDP-1612/621513
3. PJDP-1619/621520
4. AFS-68
5. DS-1059
6. DS-1077
7. DS-1156
8. DS-1158
9. DS-1188
10. IS-3222
11. SOR-8586
12. IC-4852
13. SH-1488
14. SH-1813
15. S-652
16. SSG 59-3
17. GFS 6
18. GAFS 12
19. CSV 33-MF
20. CSV 27
21. CSV 41
22. Gundari
23. Makari Jowar
24. GUNDLOPET
25. CSV 46F
26. SRF-407
27. SRF-420
28. SRF-421
29. SRF-422
30. SRF-423
31. DSF-219
32. DSF-222
33. DSF-224
34. DSF-226
35. GFS 4

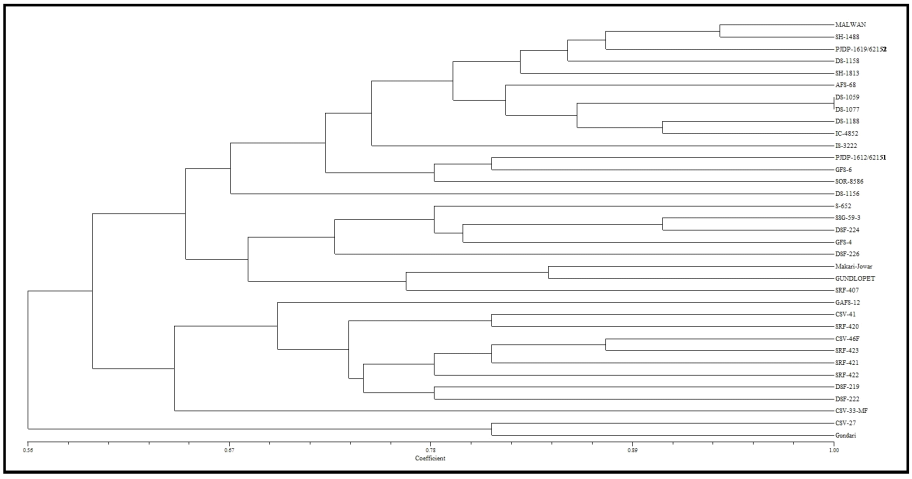

Fig. 2. Dendrogram showing clustering of thirty-five sorghum genotypes constructed using UPGMA based on Jaccard's similarity co-efficient obtained from SSR based PCR analysis.

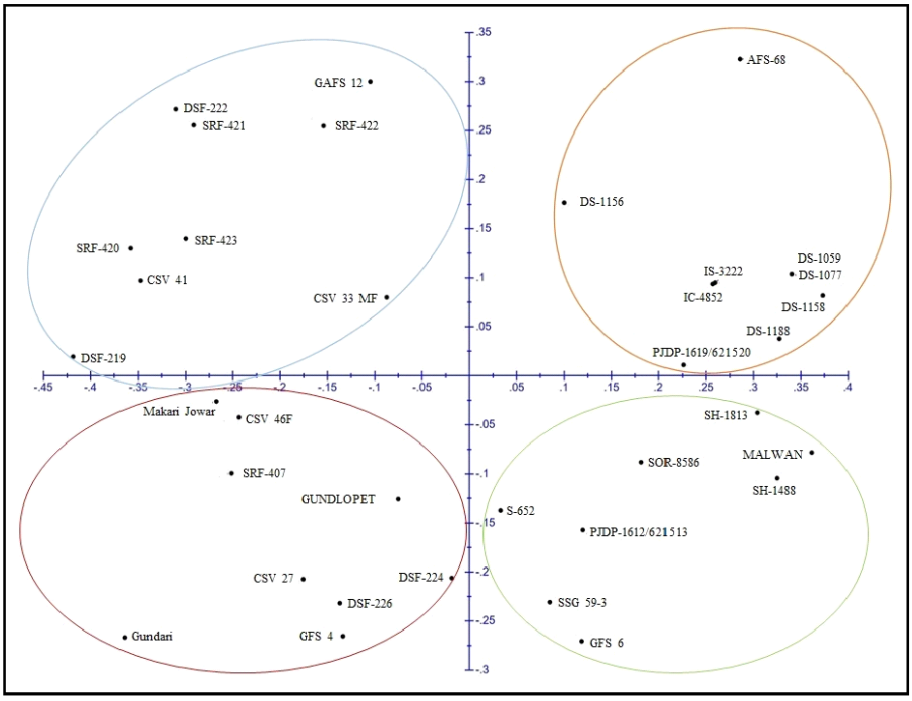

Fig. 3. Two-dimensional (2D) plot of PCoA using SSR based primers.

with fifteen genotypes *viz*., Malwan, SH-1488, PJDP-1619/621520, DS-1158, SH-1813, AFS-68, DS-1059, DS-1077, DS-1188, IC-4852, IS-3222, PJDP-1612/621513, GFS 6, SOR-8586 and DS-1156, Cluster $A_{1:2}$ include eight genotypes *viz*., S-652, SSG 59-3, DSF-224, GFS 4, DSF-226, Makari jowar, GUNDLOPET and SRF-407. The sub cluster A_2 includes cluster $A_{2:1}$ with nine genotypes *i.e.*, GAFS 12, CSV 41, SRF-420, CSV 46F, SRF 423, SRF-421, SRF-422, DSF-219 and DSF-222, while cluster $A_{2:2}$ includes only one genotype CSV 33 MF. The cluster B subdivided into sub clusters B_1 and B_2 with CSV 27 and Gundari, respectively.

Grouping of genotypes also assessed by principle coordinate analysis (PCoA) method. Principle Co-ordinate Analysis (PCoA) carried out using DARwin (version 6.0). In the PCoA (Principle coordinate analysis) plot derived from the SSR genotyping data, (which indicates that five principle coordinates), it can be observed that AFS-68 and GFS 6 are placed farthest in the first coordinates (X-axis). In contrast, DSF-219 and DS-1158 put farthest in the second coordinates (Y-axis) in Figure 3. The principle coordinate analysis (PCoA) results (Fig. 3) coincided with the results of cluster analysis. The clustering pattern of 2D plot of PCoA analysis was following the

dendrogram clustering pattern although some genotypes have been diverted on the PCoA plot.

CONCLUSION

In the present investigation, the diversity of thirty-five genotypes of forage sorghum was estimated through genetic and molecular data (*i.e.,* SSR marker). A total of thirteen distinct clusters were formed through D^2 analysis whereas, two main clusters (A and B) with two sub clusters A_1 ($A_{1:1}$ and $A_{1:2}$) in the main cluster A, while two sub clusters B_1 and B_2 in the main cluster B were formed through NTSYSpc version 2.20 programme, which revealed that grouping of genotypes through Mahalanobi's D^2 analysis was not utterly similar to a grouping of genotypes based on the molecular data through NTSYSpc version 2.20 programme. It may be due to the gene concerned with morphological traits is stage-specific, while, molecular analysis of the genome represents evolutionary variation that may be functional or non-functional. Based on combined results for genetic and molecular diversity (using SSR markers) estimates, genotype "Gundari" was found to be distinct from other genotypes and can be exploited to harness their unique features in breeding programmes.

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