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Genetic diversity analysis of sorghum (*Sorghum bicolor* L. Moench) genotypes for drought tolerance using SSR markers

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Abstract

Sorghum (*Sorghum bicolor* (L.) Moench) is considered as a model species for drought tolerance due to its inherent drought tolerant characteristics. The accessions were grouped into 27 major clusters elucidating a high level of genetic diversity which will be useful for identifying suitable genotypes for drought tolerance breeding. The best characterized form of drought tolerance during crop growth is the non-senescence or "stay-green" trait. Genetic diversity analysis carried out with 100 sorghum genotypes for drought tolerance using 13 stay-green specific polymorphic SSR markers revealed high level of polymorphism among the genotypes. A total of 56 scorable alleles were generated of which 55 were polymorphic. The number of alleles produced by different primers ranged from two to seven with an average of 4.0 alleles per primer. The polymorphic loci clearly discriminate all the genotypes. The similarity coefficients based on 13 SSR markers ranged from 0.02 to 1.00. Among the 100 accessions three genotypes *viz*., IS 29389, IS 29393 and IS 29496 and two other genotypes IS 23392 and IS 23397 showed the highest similarity index (1.00), and the genotypes IS 22005 and IS 24693, showed the least similarity index (0.02). The dendrogram, which classified the hundred sorghum accessions into twenty-seven major clusters and discriminated the drought tolerant genotypes with susceptible genotypes. Cluster analysis categorized the drought resistant and drought susceptible genotypes in to separate clusters. Most of the genotypes in the cluster IV had the better stay-green score. The stay-green specific genes may be present in most of these genotypes and classified as drought tolerant. In general, the genotypes B 35, IS 22212, IS22335, IS 22697, IS 29323, IS 22243, IS 23418, IS 22794, IS 21756 and IS 22339 exhibited drought tolerance phenomenon and were grouped as drought tolerant under molecular level of genetic diversity.

Keywords: Genetic diversity, *Sorghum bicolor*, drought tolerance, stay-green, SSR markers

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is one among the five major cereals of the world, being grown extensively in tropical and subtropical environments. The amount of genetic variability available in sorghum is very high. Sorghum is endowed with high variability due to its wide range of adaptation in tropical and temperate climates and free gene exchange among various races [1]. Drought acts as a major limiting factor in agricultural production. Improving drought tolerance is an important objective in many crop breeding programs. Drought response in sorghum has been characterized at both pre- and post-flowering stages. Drought that occurs during the post-flowering stage of crop growth and is not relieved is often referred to as terminal drought. Post-flowering drought adaptation in sorghum is associated with the stay-green phenotype [2-4]. The character that is associated with terminal drought tolerance is "stay–green". The stay-green trait is associated with the functional green leaf area and the genotypes possessing this complex trait maintain more photosynthetically active leaves compared with genotypes not possessing it. Drought stress during the post flowering stage needs serious consideration because the negative impact of post-flowering drought on yield can be very drastic. Variations in sorghum for this trait can be gainfully utilized for its genetic improvement. Molecular markers are considered such an efficient powerful tool for the assessment of genetic relationships. Because of its global socio-economic importance there has been substantial interest in characterizing the levels of genetic diversity present within the sorghum using both phenotypic and molecular markers [3, 5-10]. Simple Sequence Repeat (SSR)

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diversity has been used as a successful tool in genotyping and studying the genetic diversity of many plant species because of their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage over other DNA markers such as RFLPs, RAPDs or AFLPs [5, 6, 8]. This study was an attempt in this direction, where the efforts were directed towards assessing the genetic diversity available in sorghum germplasm for drought tolerance at molecular level using stay-green specific SSR markers which will elucidate the extent of genetic diversity for drought tolerance and identify the parental accessions for further breeding programme.

Materials and methods

Sorghum genotypes

The experimental material for this study comprised of 100 accessions of sorghum maintained by the Department of Millets, Tamil Nadu Agricultural University, Coimbatore, which included local landraces adapted to different agro-climatic regions of Tamil Nadu and different geographic locations of India besides accessions from other countries (Table 1). The genotypes showed wide range of variability for stay-green and other drought tolerance traits. In this study genotypes B 35 and CO 26 were used as resistant and susceptible checks, respectively.

Phenotypic screening

The phenotypic screening of sorghum genotypes for drought tolerance was done by raising all the 100-genotypes in a randomized block design (RBD) with two replications during the post-rainy season (November to March) of 2007-08. This season at Coimbatore is ideal for evaluating the expression of adaptive traits for terminal drought conditions, as the crop is dependent entirely on the stored soil moisture and undergoes a long, progressive stress under moderate evaporative demand conditions. Water stress was imposed by withholding irrigation at anthesis stage and continued till maturity. No rain was recorded from anthesis to crop maturity. The stay-green expression of individual genotypes along with the checks was estimated visually on a plot basis on a scale of 1 to 5 based on the degree of leaf and plant death at physiological maturity in the field under post-flowering drought stress. A rating of 1 indicated essentially no leaf death, while a rating of 5 corresponded to complete plant death (leaves and stem). Visual ratings of leaf and plant senescence have been demonstrated to be a reliable indicator of stay-green response [11]. The phenotypic stay green score of the genotypes is presented in Table 1.

DNA isolation, amplification and gel electrophoresis

Leaf samples were collected from 15 days old plants and total genomic DNA was isolated from the sorghum genotypes following modified CTAB DNA extraction protocol [12]. The SSR amplification was performed with a thermal cycler in a 15 µl solution containing 20 ng template DNA, 1.0 ml of 0.5 mM of each primer (forward and reverse), 0.75 units of *Taq* DNA polymerase, 0.6 ml of 10 mM of each dNTP, 1 x reaction buffer and distilled de-ionized water. The PCR conditions were as follows: an initial denaturation step of 2 min at 94^{o}C, followed by 35 cycles at 94^{o}C for 1 min, 52^{o}C-61^{o}C (depending upon the primer pair) for 1 min, 72^{o}C for 1 min. The final PCR cycle of 72^{o}C for 7 minutes. The PCR products were separated by 3% agarose gel electrophoresis in 1x TBE buffer at 100v for 3 hours. The Ethidium bromide pre-stained gels were documented using Alpha Imager ^{TM} 1200. (Alpha Innotech Corporation, California, USA). The list of primers [13] used to survey the genotypes are presented in Table 2.

Data analysis

All the genotypes were scored for presence and absence of the SSR bands. The data were entered in to a binary matrix as discrete variables (1) for the presence of the amplification product or band and (0) for the absence of the band and this matrix were subjected to further analysis. The scores of individual bands were used to create a data matrix [14]. The similarity index (SI) values were computed as the ratio of number of similar bands to the total number of bands in pair wise comparison of the genotypes. A Dendrogram was constructed based on Jaccard's similarity coefficient [15] with Unweighted Pair Group Method and Arithmetic Average analysis (UPGMA) [16] using the NTSYS-pc version 2.1 Software [17]. To measure the informativeness of the markers, the Polymorphism Information Content (PIC) for each SSR marker was calculated according to the formula: PIC = 1-Σpi^2, where *pi* is the frequency of the i^{th} allele [18].

Results and discussion

Screening for the stay-green phenotype

Drought resistance is a complex trait affected by several interacting plant and environmental factors. Stay-green or non-senescence is an important trait associated with drought tolerance. It has been confirmed by many authors [2-4] that genotypes possessing the stay-green

Table 1. List of sorghum genotypes studied for genetic diversity for drought tolerance

S.No	Genotypes	Source	Stay green score*	S.No	Genotypes	Source	Stay green score*
1	B 35	NRCS	1.75	51	IS 25601	ICRISAT	4.50
2	CO 26	Coimbatore	4.21	52	IS 25602	ICRISAT	5.00
3	M 35-1	NRCS	3.16	53	IS 25760	ICRISAT	4.70
4	CO(S) 28	Coimbatore	3.85	54	IS 25779	ICRISAT	4.90
5	IS 21756	Sudan	2.74	55	IS 26103	ICRISAT	4.82
6	IS 21757	Sudan	3.48	56	IS 26700	ICRISAT	4.90
7	IS 22005	Maharashtra	3.24	57	IS 26742	ICRISAT	4.78
8	IS 22212	USA	2.14	58	IS 39690	ICRISAT	4.65
9	IS 22215	USSR	3.10	59	IS 26760	ICRISAT	5.00
10	IS 22233	Botswana	4.40	60	IS 27874	ICRISAT	3.95
11	IS 22243	Botswana	2.85	61	IS 27875	ICRISAT	3.85
12	IS 22244	Botswana	4.45	62	IS 29115	ICRISAT	3.78
13	IS 22248	Botswana	4.40	63	IS 29218	ICRISAT	3.85
14	IS 22251	Botswana	4.10	64	IS 29231	ICRISAT	4.10
15	IS 22334	Botswana	5.00	65	IS 29239	ICRISAT	3.95
16	IS 22335	Botswana	2.26	66	IS 29251	ICRISAT	4.24
17	IS 22339	Botswana	3.08	67	IS 29277	ICRISAT	4.15
18	IS 22349	Botswana	4.14	68	IS 29278	ICRISAT	4.25
19	IS 22360	Botswana	3.85	69	IS 29306	ICRISAT	3.90
20	IS 22697	Sudan	2.24	70	IS 29307	ICRISAT	3.54
21	IS 22764	Somalia	3.85	71	IS 29322	ICRISAT	3.68
22	IS 22765	Somalia	2.75	72	IS 29323	ICRISAT	1.98
23	IS 22794	Somalia	3.21	73	IS 29341	ICRISAT	3.45
24	IS 22959	Somalia	5.00	74	IS 29344	ICRISAT	4.00
25	IS 23158	Sudan	4.80	75	IS 29358	ICRISAT	2.45
26	IS 23390	Tanzania	5.00	76	IS 29359	ICRISAT	4.70
27	IS 23392	Sudan	4.98	77	IS 29379	ICRISAT	3.20
28	IS 23397	India	4.86	78	IS 29386	ICRISAT	3.98
29	IS 23402	India	5.00	79	IS 29389	ICRISAT	4.85
30	IS 23408	India	4.80	80	IS 29393	ICRISAT	4.78
31	IS 23418	India	2.45	81	IS 29440	ICRISAT	3.89
32	IS 23419	India	5.00	82	IS 29450	ICRISAT	3.95
33	IS 23422	India	4.89	83	IS 29451	ICRISAT	4.25
34	IS 23429	India	5.00	84	IS 29458	ICRISAT	4.15
35	IS 23430	India	4.80	85	IS 29459	ICRISAT	4.54
36	IS 23440	India	4.60	86	IS 29484	ICRISAT	3.45
37	IS 23442	India	4.25	87	IS 29487	ICRISAT	3.90
38	IS 23446	India	4.25	88	IS 29496	ICRISAT	4.76
39	IS 23453	India	4.10	89	IS 29498	ICRISAT	4.42
40	IS 23455	India	4.87	90	IS 29509	ICRISAT	4.84
41	IS 23460	India	4.42	91	IS 29515	ICRISAT	4.64
42	IS 23477	India	4.68	92	IS 29523	ICRISAT	3.45
43	IS 23590	India	4.70	93	IS 29545	ICRISAT	4.10
44	IS 24484	Ethiopia	5.00	94	IS 29554	ICRISAT	4.60
45	IS 24693	South Africa	4.95	95	IS 29573	ICRISAT	4.23
46	IS 24978	India	4.86	96	IS 29589	ICRISAT	2.20
47	IS 25004	Sudan	4.87	97	IS 29611	ICRISAT	4.60
48	IS 25071	Sudan	4.80	98	IS 29625	ICRISAT	4.50
49	IS 25098	Ghana	4.70	99	IS 29629	ICRISAT	4.20
50	IS 25400	Ghana	5.00	100	IS 29640	ICRISAT	4.45

*Phenotypic stay green score recorded on 1- 5 scale (1: All leaves natural green; 5: All leaves dried)

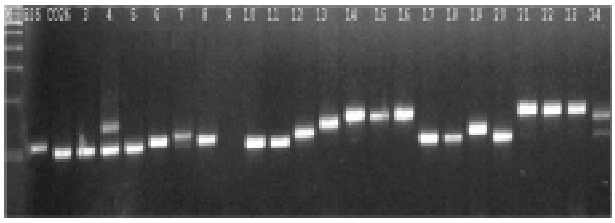
trait have a significant yield advantage under post-anthesis drought stress conditions compared with genotypes not possessing this trait. It was reported in a study with nine genotypes varying in stay-green [4] that grain yield was correlated positively with green leaf area at maturity (r=0.75**) and negatively with rate of leaf senescence (r=-0.74**), suggesting that sorghum genotypes possessing the stay-green trait have a significant yield advantage under post-anthesis drought compared with genotypes not possessing this trait. It was also reported that the stay-green did not constrain yield when water was not limiting, since no differences in grain yield were observed among eight of nine genotypes under fully-irrigated control conditions. When water was limiting during grain growth, yield accumulation in stay-green genotypes was largely dependent on photo-assimilation in the remaining green leaves [4]. Results from breeding programs in the USA (2) and Australia (19) suggest that advances in crop improvement under water-limited conditions are more likely if drought resistance traits are selected in addition to yield *per se*. Taking in to account the stay-green trait confers the resistance to post-flowering moisture stress, phenotypic screening for drought resistance in this study was carried out by scoring stay-green trait of the genotypes under induced post-flowering moisture stress. The field screening of genotypes for post flowering drought tolerance revealed that the genotypes expressed more variability for stay-green trait and the stay-green score ranged from 1.75 to 5.0 with a mean of 4.12. Similar trend of variability for stay-green among one hundred and forty six sorghum genotypes was also reported earlier [20]. In this study, eleven genotypes were identified as drought resistant with a stay-green score of below 3.00. This includes the drought resistant check variety B 35 with other genotypes *viz.,* IS 29323, IS 22212, IS 29589, IS 22335, IS22697, IS 23418, IS 29358, IS 21756, IS 22765 and IS 22243 collected from different geographical locations. These genotypes possessed green and non-senescent leaves during post flowering and grain filling stage and should have a yield advantage as compared to drought susceptible senescent genotypes. Other twenty three genotypes with stay-green score of 3.01 to 4.00 were identified as moderately drought resistant genotypes, while sixty three genotypes were identified as drought susceptible with a score of more than 4.01. This indicated the existence of enormous genetic variability for drought resistance trait in sorghum germplasm selected for these studies.

SSR Analysis

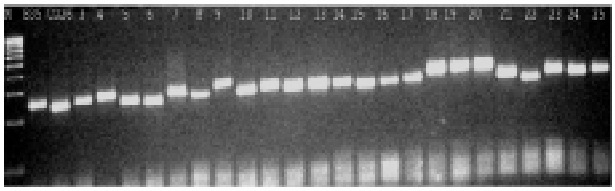
The one hundred sorghum genotypes were surveyed with 16 microsatellite markers for their distribution, informativeness and polymorphism for the assessment of genetic diversity for drought tolerance among them. Among the 16 primers, 13 were polymorphic and produced scorable, unambiguous bands. A total of 56 scorable alleles were generated by these 13 primers of which 55 were found to be polymorphic. In the present study a high level of polymorphism (91.4%) was observed among the 100 sorghum genotypes. The number of alleles produced by different primers ranged from two to seven with an average of 4.0 alleles per primer. Earlier studies with twenty-two accessions of *Sorghum bicolor* from Ethiopia, China and USA using SSR markers revealed high level (95.4%) of polymorphism and similar number of alleles (4.5) per primer [21]. The mean number of alleles per locus and allelic size generated in this study was in close agreement with earlier studies on sorghum characterization [22-24]. Thus the results clearly indicated that the sorghum genotypes could be distinguished using SSR primers. The maximum number of amplified product (seven) was observed in the profiles of primer Xtxp 43 while, the minimum number of amplified product (two) was observed in the profiles of primer Xtxp 59.The SSR banding pattern of the sorghum genotypes using the primer Xtxp 43 and Xtxp 285 are shown in the Fig. 1. The polymorphism information content (PIC) as a relative measure of informativeness was calculated for 13 SSR markers. The PIC was the highest for the SSR primer Xtxp 43 (0.914) and lowest for the primer Xtxp 59 (0.427) with a mean of 0.738 (Table 2). The higher the PIC value, the more informative is the SSR markers. These are in close agreements with earlier reports [25, 26] in sorghum and barley. Since the higher PIC value indicates more informativeness for genotype discrimination and diversity studies, these primers would be of great value in characterization of sorghum germplasm for drought tolerance and also for fingerprinting sorghum varieties.

Genetic relationship among sorghum genotypes

The binary data from the polymorphic primers were used for computing Jaccard's similarity indices. The similarity coefficients based on 13 SSR markers ranged from 0.02 to 1.00. Five genotypes *viz*., IS 29389, IS 29393 and IS 29496, IS 23392 and IS 23397 showed the highest similarity index (1.00) and the genotypes IS 22005 and IS 24693, showed the least similarity index (0.02). The SSR markers showed very high dissimilarity between



a. Xtxp 43



b. Xtxp 285

Fig. 1. SSR banding pattern of the sorghum genotypes

Table 2. SSR Marker investigation for 100 sorghum genotypes

S.No.	Primer name	No. of alleles	Polymorphic alleles	PIC
1.	Xtxp-43	7	7	0.914
2.	Xtxp-88	6	6	0.860
3.	Xtxp-296	5	5	0.844
4.	Xtxp-302	5	5	0.570
5.	Xtxp-295	4	4	0.710
6.	Xtxp-1	4	4	0.828
7.	Xtxp-340	4	4	0.740
8.	Xtxp-38	4	4	0.775
9.	Xtxp-8	5	5	0.814
10.	Xtxp-208	3	3	0.713
11.	Xtxp-23	3	3	0.723
12.	Xtxp-285	3	3	0.677
13.	Xtxp-59	3	2	0.427

the sorghum genotypes used in this study with a mean similarity index of 0.36. Higher the dissimilarity between the genotypes, better the scope to identify drought tolerant genotypes as against susceptible one. Drought tolerant genotypes with high similarity percentage could be useful as donors in hybridization programme for the transfer of the drought tolerance traits. SSR alleles within the local land races could be selected through drought stressed environments, thereby reducing genetic variability among them. Using SSR markers, reduction in genetic variability was detected within wild barley grown in drought conditions indicating that stress conditions may lead to selection [27].

Cluster analysis

The cluster analysis based on the similarity coefficients using UPGMA grouped in to twenty seven major clusters. The composition of clusters (Table 3). Cluster VII comprised maximum number of genotypes (12) followed by cluster IV (10), cluster XV (9) cluster XVIII (8), cluster XVII (7) and cluster XVI (5). Clusters VIII, XI, XIV, XIX and XX had four genotypes each and XIII and XXVI had three genotypes each. Clusters II, V, IX, X, XII, XXI, XXIII, XXIV and XXV had two genotypes each. Clusters I, III, VI, XXII and XXVII had one genotype each (Fig. 2).

Clustering pattern and drought response

The genotypes, which were grouped in to different clusters, were compared with their visual stay-green ratings made during the field level studies to know the level of expression of stay-green genes under molecular level. The cluster IV with the genotypes B 35, IS 22212, IS22335, IS 22697, IS 29323, IS 22243, IS 23418, IS 22794, IS 21756 and IS 22339 and cluster number XXI with IS 29589 and IS 29358 had better stay-green score. Though the geographic origin of these genotypes is different they were found to be grouped in single cluster due to their close genetic similarity. The stay-green specific genes may be present in most of these genotypes and classified as drought tolerant. These sorghum accessions may be suitable for growing under drought prone areas of rainfed tracts and may also be used as donor for developing high yielding drought resistant stay-green varieties through recombination breeding. Most of the genotypes in the clusters I, II, III, V, VI, VIII, IX, X, XI, XII, XIII, XIV, XVI, XVII, XVIII, XIX, XX, XXII, XXIII, XXIV, XXV, XXVI and XXVII appeared to have moderate level of stay-green. These genotypes may have moderate level of expression of stay-green specific genes and can be useful as parents for general breeding purposes in moderate stress environments. The twelve genotypes mostly with senescent reaction to drought in cluster VII which include susceptible check genotype CO 26 and cluster XV with nine drought susceptible genotypes were found to be drought susceptible where there may be absence of stay-green specific genes resulting in low level of stay-green expression and may be suitable for irrigated ecosystem only. The current study included a wide range of genotypes representing more than one location but of course with same geographic origin. Hence the clustering pattern of the accessions showed that though the genotypes represent a single geographical origin, exhibited greater molecular diversity among them. This

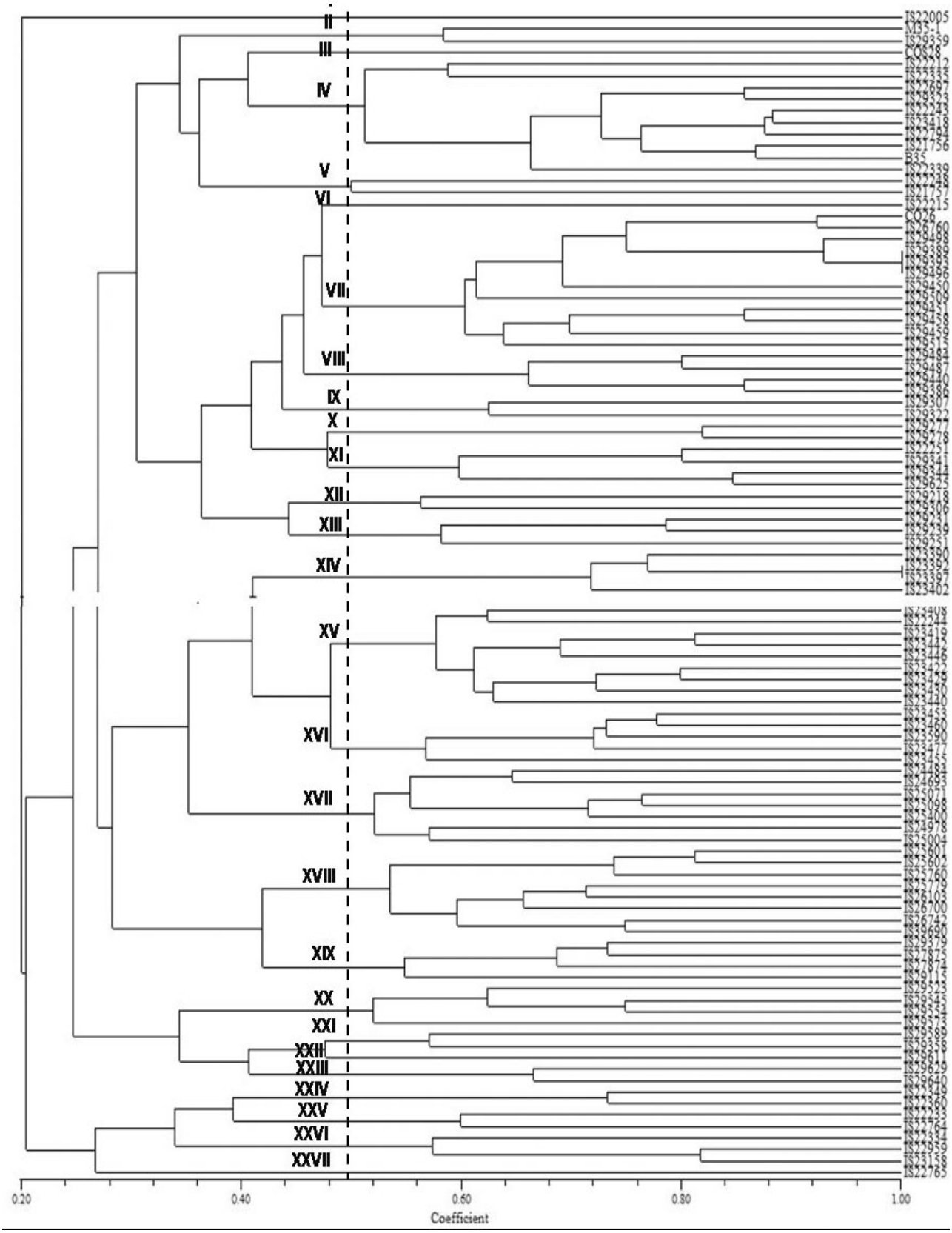


Fig. 2. Genotypic dendrogram depicting the clustering pattern among 100 sorghum accessions constructed using 13 stay-green specific SSR primers

Table 3. Cluster composition of 100 sorghum genotypes based on SSR analysis.

Cluster no.	No. of genotypes	Name of genotypes
I	1	IS22005
II	2	M35-1, IS29359
III	1	CO(S)28
IV	10	IS22212,IS22335,IS22697, IS29323,IS22243,IS23418, IS22794,IS21756,B35,IS22339
V	2	IS22248, IS21757
VI	1	IS22215
VII	12	CO26,IS26760,IS29498,IS29389, IS29393,IS29496,IS29450, IS29509, IS29451,IS29458,IS29459,IS29515
VIII	4	IS29484,IS29487,IS29440,IS29386
IX	2	IS29307,IS29322
X	2	IS29277,IS29278
XI	4	IS22251,IS29341,IS29344,IS29625
XII	2	IS29218,IS29306
XIII	3	IS29231,IS29239,IS29251
XIV	4	IS23390,IS23392,IS23397,IS23402
XV	9	IS23408,IS22244,IS23419, IS23442,IS23446,IS23422, IS23429,IS23430,IS23440
XVI	5	IS23453,IS23460,IS23590, IS23477,IS23455
XVII	7	IS24484,IS24693,IS25071, IS25098,IS25400,IS24978,IS25004
XVIII	8	IS25601,IS25602,IS25760, IS25779,IS26103,IS26700, IS26742,IS39690
XIX	4	IS29379,IS27875,IS27874,IS29115
XX	4	IS29523,IS29545,IS29554,IS29573
XXI	2	IS29589,IS29358
XXII	1	IS29611
XXIII	2	IS29629,IS29640
XXIV	2	IS22349,IS22360
XXV	2	IS22233,IS22764
XXVI	3	IS22334,IS22959,IS23158
XXVII	1	IS22765

is in agreement with the findings of earlier workers with sorghum land races collected from Tamil Nadu [28]. The stay-green specific primers used in this study sorted the genotypes based on their stay-green trait or the level of drought tolerance. The grouping of genotypes in to different clusters based on their reaction to post flowering moisture stress using molecular marker under this study has great utility in genetic diversity studies. The current study elucidated the presence of high level of genetic variability in sorghum germplasm which would be very useful for identifying suitable donor in breeding for drought tolerant varieties in sorghum. Therefore molecular markers like SSR could serve as a basic tool for the genetic diversity analysis and also for fingerprinting of closely related varieties and tagging of drought related traits and linkage mapping in sorghum.

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