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Genetic diversity among elite sorghum (*Sorghum bicolor* L.) accessions genotyped with SSR markers to enhance use of global genetic resources

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Abstract
Analysis of genetic diversity, selection of parents with diverse genetic base having contrasting phenotype is an important step in developing mapping populations for QTL detection, association mapping and MAS studies. To elucidate the diversity among Marathwada (Maharashtra) region cultivated sorghum accessions we studied genetic diversity in 20 sorghum accessions using SSR markers. The polymorphism information content (PIC), a measure of gene diversity present in the selected population, varied from 0.38 to 0.72 with an average of 0.55 and was significantly correlated with number of alleles. Clustering analysis based on the unrooted phylogeny using neighbour-joining (NJ) method, bootstrapping of the data (10,000 permutations) as implemented in POWERMARKER grouped the 20 accessions into three clusters and grouping was in good agreement with biological status and some important traits of sorghum accessions. The SSR loci msbCIR306, Xtxp321, msbCIR329, Xcup02 and Xisep0310 were rich in allelic diversity exhibiting highest PIC value.

Keywords: Sorghum, genetic diversity, SSR markers, polymorphic information content (PIC)

Introduction
Sorghum [*Sorghum bicolor* (L.)] is a genus with many species and subspecies, and there are several types of sorghum, including grain sorghums, grass sorghums (for pasture and hay) and sweet sorghums (for syrups). Sorghum has the high nutritional values and is grown as dietary staple food for more than 500 million people in more than 30 different countries. It is also important source of nutraceuticals due to its high content of all type of antioxidant phenolic compounds [1-2]. Sorghum is well known for its better agronomic performance, that is, adaptability to grow in varying environmental conditions. It is known that sorghum is a drought tolerant, heat tolerant, and can grow in high altitudes and saline–alkaline soil. This is because sorghum has a well-developed deep and branched root system with a high root to leaf ratio, and the leaves are covered by wax and can also roll themselves in response to drought stress [3].

As the marathwada region of Maharashtra, India has low rainfall which adversely affects plant growth and development, resulting in substantial losses to the yields of highly important crops. So a good knowledge and usage of this genetic resource in sorghum accessions is highly vital for improving the present and future crop quality. The major challenge in front of mankind is to fulfil the demand of growing population by producing large amount of food in less area. It is a tough situation for scientist to find out new genotypes of food crops which can adapt themselves according to adverse change in nature. For that the current and future need is to develop the high yielding drought resistance sorghum variety for farmer's benefits.

Analysis of genetic diversity can be very useful for sorghum breeding programs, in which understanding the relatedness between accessions is essential to define breeding strategies and to identify polymorphic superior parents for the development of new cultivars [4-9]. Several strategies have been used to access genetic diversity in many crop species [10-14] based on agro-morphological, molecular analysis, geographical locations and biochemical differences among accessions. In the past years, estimation of genetic diversity in cultivated sorghum based on phenotypic traits was carried out to identify the polymorphic superior parents for breeding

purpose [15-17]. Even though phenotypic characterization provides a range of information about the genetic variability among accessions in a germplasm bank, the effects of environment, genotype-by-environment interaction, and human errors during measurement also contribute to the observed differences [18-19]. Thus, some previous studies have reported that the use of DNA based molecular markers could be advantageous to analyzed the genetic differences among accessions in various crops [20-22].

The above reports revealed that use of molecular markers in genetic diversity analyses has an promising advantages over the phenotypic characterization, as the molecular markers are not influenced by the environmental effect. Different molecular markers have been widely used to access genetic diversity in sorghum such as SNPs, SSR RAPD and AFLP [23-26]. In this study, SSR molecular markers were used which are widely distributed on sorghum genome to assess genetic diversity in 20 sorghum accessions specific to Marathwada region of Maharashtra. All the sorghum germplasm were collected from ICRISAT Patancheru, Hyderabad, India. Simple sequence repeat (SSR) markers are particularly attractive for studying genetic differentiation in the plant and animal genomes because of their abundance in the genome [27-28]. In the present study the 20 sorghum accessions specific to Marathwada, Maharashtra region consisting of advance/ improved cultivar, traditional cultivar/landrace, breeding/ research material were screened for genetic diversity in order to exploit the use of genetic resources for crop breeding and mapping studies.

The aim of this study is to characterize sorghum accessions as an initiative step in testing, the applicability of molecular markers for choosing parents of high yielding sorghum hybrids, eight sorghum SSR markers evenly distributed on sorghum genome were used to analyses the genetic variation among different germplasm accessions. The objectives of this study are:

1. To determine the genetic diversity of Indian cultivated sorghum accessions.
2. To know the diversity of sorghum accessions using SSR markers.

Materials and methods

1. Plant materials

The 20 sorghum accessions specific to Marathwada, Maharashtra region were procured from International Crop Research Institute for Semi-Arid and Tropics (ICRISAT) gene bank and evaluated for genetic diversity analysis, their biological status and line grouping are listed in Table 1.

Table 1: List of sorghum germplasm accession used in this study with their biological status.

S. No.	Accession ID	Code No.	Biological Status	Collection site/Country of origin (India)
1	PJ 7R	SG1	Advanced/Improved cultivar	Parbhani; Parbhani district
2	Kharif nilwa phulmain	SG2	Traditional cultivar/Landrace	Aurangabad; Aurangabad district
3	Maldandi devgaon	SG3	Traditional cultivar/Landrace	Aurangabad; Aurangabad district
4	Dagri devgaon	SG4	Traditional cultivar/Landrace	Aurangabad; Aurangabad district
5	Bedri shivar	SG5	Traditional cultivar/Landrace	Aurangabad; Aurangabad district
6	Dagri mehagaon	SG6	Traditional cultivar/Landrace	Aurangabad; Aurangabad district
7	PJ3K	SG7	Breeding/Research material	Parbhani; Parbhani district
8	PJ4K	SG8	Breeding/Research material	Parbhani; Parbhani district
9	PJ5K	SG9	Breeding/Research material	Parbhani; Parbhani district
10	Tuljapuri degloor	SG10	Traditional cultivar/Landrace	Nanded; Nanded district
11	Dukri degloor	SG11	Traditional cultivar/Landrace	Nanded; Nanded district
12	Manthi palsa	SG12	Traditional cultivar/Landrace	Nanded; Nanded district
13	IS 17959	SG13	Traditional cultivar/Landrace	Hingoli; Parbhani district
14	IS 17960	SG14	Traditional cultivar/Landrace	Hingoli; Parbhani district
15	IS 17961	SG15	Traditional cultivar/Landrace	Hingoli; Parbhani district
16	IS 17962	SG16	Traditional cultivar/Landrace	Nanded; Nanded district
17	IS 17963	SG17	Traditional cultivar/Landrace	Nanded; Nanded district
18	PJ 1R	SG18	Breeding/Research material	Parbhani; Parbhani district
19	PJ 2R	SG19	Breeding/Research material	Parbhani; Parbhani district
20	Dagadi	SG20	Traditional cultivar/Landrace	Parbhani; Parbhani district

2. DNA preparation

Seeds of 20 accessions were grown on experimental research field of MGM CABT Gandheli, Aurangabad during Rabi-December 2019. Genomic DNA was extracted from leaves of 20 days old seedlings using modified CTAB protocol. Total DNA was extracted from 0.2g fresh weight of leaves that yielded about 1555.2-3000 ng.µl-1 total DNA. A 50 ng.µl-1 diluted DNA of these total extracts DNA was consecutively used for PCR amplification.

3. SSR markers

Eight primers pairs (SSR markers) were used for genotyping (Table 2) were part of a sorghum SSR kit[29] (http://sorghum.cirad.fr/SSR_kit), which provides reasonable coverage across the sorghum nuclear genome. SSR (simple sequence repeats) are reliable and informative PCR based molecular markers that are highly polymorphic, co-dominant, abundant and uniformly dispersed in the plant genome.

Table 2: Characteristic of the eight SSR loci analyzed.

S. No.	Marker name	Forward primer	Reverse primer	Allele size	Ch. No
1	Xtxp321	TAACCCAAGCCTGAGCATAAGA	CCCATTCACACATGAGACGAG	192-252	SBI08
2	MsbCIR329	GCAGAACATCACTCAAAGAA	TACCTAAGGCAGGGATTG	109-117	SBI05
3	MsbCIR306	ATACTCTCGTACTCGGCTCA	GCCACTCTTTACTTTTCTTCTG	120-124	SBI01
4	Xtxp057	GGAACTTTTGACGGGTAGTGC	CGATCGTGATGTCCCAATC	223-257	SBI06
5	Xcup02	GACGCAGCTTTGCTCCTATC	GTCCAACCAACCCACGTATC	192-204	SBI09
6	Xisep0310	TGCCTTGTGCCTTGTTTATCT	GGATCGATGCCTATCTCGTC	164-219	SBI01
7	gpsb151	ATACCAAGTTTCCTTTACCT	GTTGGGGGAGAGTTTT	106-128	SBI04
8	Xcup14	TACATCACAGCAGGGACAGG	CTGGAAAGCCGAGCAGTATG	211-225	SBI03

4. PCR Amplification

Eight sorghum SSR markers, after its optimization and screening were selected and used. The microsatellite PCR was performed for all accessions in a volume of 20µl reaction that contained 40-50ng of template DNA (2.0µl), 10X PCR buffer with 25mM $MgCl_2$ (2.0 µl), 2mM $dNTP_s$ (2.0 µl), 10µM forward and reverse primer (2.0µl), 1µl Taq DNA Polymerase (1U/µl) and Nanopore water. In regards to maximum yield and specificity, annealing temperature (T_m) and cycling times were rearranged for each primer pair. Amplification was performed in an APPLIED BIOSYSTEM thermal cycler with an initial denaturation at 94^0C for 5 mins followed by 34 cycles at 94^0C for 30sec, 55-65^0C for 30 sec (depending on primer) and 72^0C for 1min, followed by a final extensions at 72^0C for 7mins.

5. Gel Electrophoresis and detection

In order to obtained accurate fragment size and to identify small polymorphic differences between fragments, PCR products were resolved on 3.5% agarose gel in 1X TAE buffer. The amplified Polymerase chain reaction (PCR) reactions were mixed with Bromophenol blue dye prior to loading and the samples loaded in wells were run at 200volt. The gel was visualized under UV light using Gel documentary unit and the fragment sizes for each marker were determined. Based on the expected product size, the size of most intensely amplified bands around the expected product size for each marker was identified using standard molecular weight size marker (50/100bp DNA ladder; NEB (ILS) Company). The band appeared in specific primers were scored in the form of matrix with different type of alleles which indicate the different allelic forms of the same marker.

6. Statistical analyses

For a set of sorghum accession, genetic diversity parameters such as number of alleles per locus, heterozygosity, major allele frequency, gene diversity and polymorphic information content (PIC) for each marker was estimated using program POWERMARKER version 3.25 [30]. Allele frequency represents the frequency of particular allele for each marker. Heterozygosity is a proportion of heterozygous individual in the population. Polymorphic information content (PIC) that represent the amount of polymorphism within a population. For the construction of phylogenetic tree Nei's distance [31] was calculated and used for the construction of unrooted phylogeny using neighbour-joining (NJ) method, bootstrapping of the data (10,000 permutations) as implemented in POWERMARKER, and MEGA 6.0 was used to visualize the Tree [32]

Result

Amplification product and estimation of genetic diversity

In the present study the eight SSR markers [29] (http://sorghum.cirad.fr/SSR_kit) were used for diversity study in twenty accessions of sorghum show amplification and polymorphism. All the markers produce all expected SSR allele size amplicons were used for analysis of genetic diversity among the twenty accessions of elite sorghum.

In total 24 alleles were detected in 20 germplasm accessions. The number of alleles per loci varied from 3 to 5 with an average of 4 alleles per locus. The highest number of alleles (5) was recorded for the loci Xtxp321 and msbCIR306 (5 alleles) whereas, the lowest number of allele (3) was detected for a two markers viz., Xcup02 and msbCIR329 (Table3 and 4). PIC value represents the relative informativeness of each marker and in the present study, the average PIC value was found to be 0.55 was comparable to past and earlier reports for sorghum and rice [33-35]. PIC values was found to be ranged from 0.38 to 0.72 where 0.38 PIC value indicated presence of three allele per locus. Out of eight polymorphic markers, six (75.0%) markers were highly informative (PIC ≥ 0.5), two markers (25%) were reasonably informative (PIC = 0.25–0.5) and no one markers was found to be less informative (PIC ≤ 0.25). No heterozygosity was found across 147 accessions which may be due to autogamous nature of rice. The SSR loci msbCIR306, Xtxp321, msbCIR329, Xcup02 and Xisep0310 were rich in allelic diversity exhibiting highest PIC value. Expected heterozygosity or Gene diversity computed according to Nei (1973) [31] and varied from 0.41 to 0.76 with an average of 0.61 (Table 3). The major allele frequency across 20 accessions was ranged from 0.35 to 0.75 with the mean major allele frequency was 0.51. Thus, the 20 sorghum accessions used in this study have wide genetic diversity and are good candidates for candidate gene specific association studies of complex trait such as yield and for using parents in plant breeding programme.

Table 3: Mean diversity analysis of 20 germplasm accessions using eight SSR markers.

S. No.	Diversity traits	Number	Range	
1	Total no. of alleles	24	Maximum	Minimum
2	Mean no. of alleles per locus	4	5	3
3	Mean Major allele frequency	0.516	0.75	0.35
4	Mean gene diversity	0.61	0.76	0.41
5	Mean PIC	0.555	0.72	0.38

Distance based approach which is based on calculating pair wise distance matrix was computed by calculating a dissimilarity matrix using a Nei distance (1973) and used for the phylogenetic tree construction by Neighbour-Joining (NJ) method, implemented in POWERMARKER version 3.25 [29] and the tree was viewed using MEGA V 6.0. A neighbour-joining (NJ) tree was constructed based on genetic distance (Figure 1). The group I in NJ tree consists of most of the traditional landraces whereas the II group consists of mostly breeding material. Further in group 1 and 2, the accessions were classified into sub-sub groups based on their origin and types. Similar observations were arrived by many investigators in different crops [35-37].

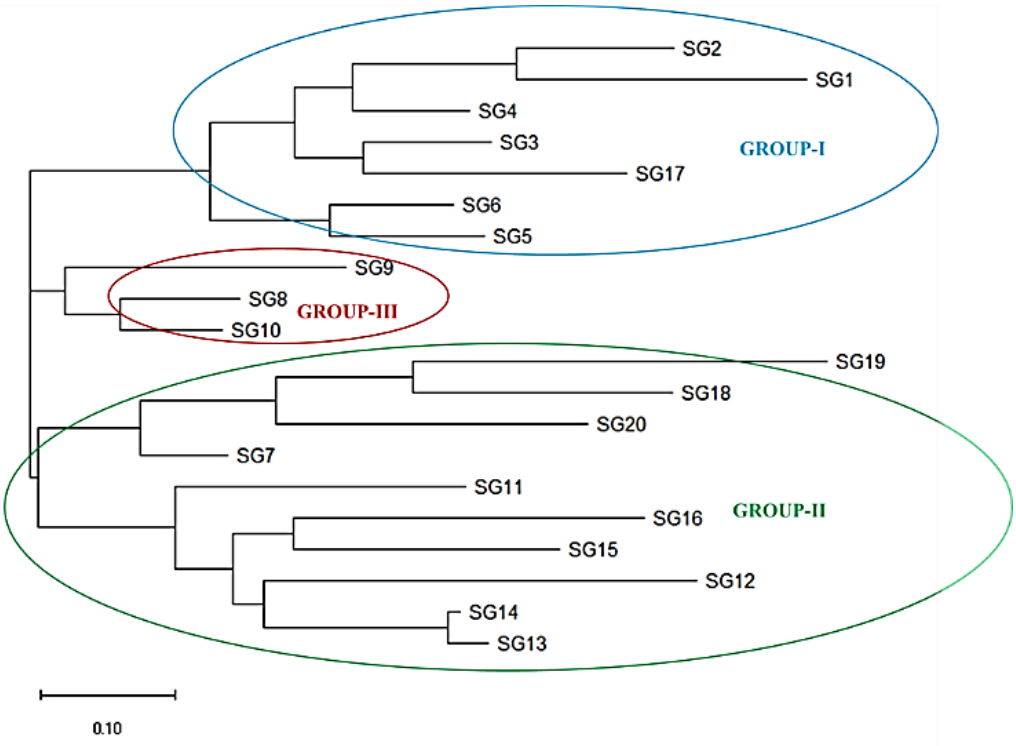

Fig 1: Neighbor-joining (NJ) tree of 20 Sorghum accessions constructed using POWERMARKER software.

Table 4: Details of SSR loci used for genotyping in the 147 rice accessions and their genetic diversity parameters using power marker V 3.25 program.

S. No.	Marker	Chr. No.	Minimum molecular weight	Maximum molecular weight	Major Allele Frequency	Allele No	Gene Diversity	PIC
1	Xtxp321	8	191	210	0.40	5	0.705	0.6533
2	msbCIR329	10	112	117	0.55	3	0.585	0.5129
3	msbCIR306	1	120	124	0.35	5	0.760	0.7224
4	Xtxp057	9	240	255	0.75	4	0.415	0.3894
5	Xcup02	6	200	204	0.55	3	0.585	0.5129
6	Xisep0310	2	195	215	0.50	4	0.615	0.5444
7	Xcup14	3	211	225	0.41	5	0.690	0.63
8	gpsb151	4	106	128	0.44	5	0.720	0.71

Discussion

The germplasm of various crops is available worldwide; their utilization is an important task for the crop improvement to develop a hybrid variety having higher yield potential and to sustain in adverse environment conditions. Hence genetic diversity assessment is a key determinant of germplasm utilization in crop improvement. Analyses of genetic diversity in the collection of sorghum accessions provided important information to define breeding strategies and to identify superior parents for the development of new sorghum cultivars focusing on various biotic abiotic stresses. Also the assessment of diversity and analysis of population structure represents important information for genetic analysis and identification of quantitative trait loci by means of association mapping [38]. In the present study the clusters obtained by the molecular diversity analysis were more consistent with the some important characteristics traits and the historic background of the sorghum accessions. Total 20 accessions were grouped into three main clusters and sub-sub groups based on the molecular marker data generated from evenly distributed SSR markers on the whole genome of sorghum.

Also there are some important findings were published by the authors on diversity analysis in sorghum accession from different origins. The genetic diversity within small sets of Guinea-race sorghum accessions has been analyzed in the past studies using different molecular markers such as, SSR markers [39], random amplified polymorphic DNA (RAPD) markers [40-41] and restriction fragment length polymorphism (RFLP) markers [42].The main focused of these studies was on the analyses of variation between the different races of sorghum. Employing SSR markers to analyze the gene diversity observed in this study (Mean PIC = 0.61) is similar to the diversity value (0.40, 0.62, 0.58) reported by[43-45], respectively. The genetic variation among five Guinea-race sorghum accessions [39], revealed an observed heterozygosity of 0.089, with average expected gene diversity of 0.224 which was much lesser than the gene diversity obtained in the current study using 20 sorghum accessions originated from Marathwada region of Maharashtra, India.

The polymorphic information content (PIC) of an SSR marker offers an estimate of the discriminatory power of that SSR marker by taking into account not only the number of alleles that are detected during the analysis but also the relative frequencies of those alleles[45]. An SSR marker used in this study shows a moderate discriminatory power across the 20 accessions. The SSR loci msbCIR306, Xtxp321, msbCIR329, Xcup02 and Xisep0310 were rich in allelic diversity exhibiting highest PIC value than other marker loci.

Several reports in various crop species indicated the number of alleles per locus and polymorphic information content of 4.8–14.0 and 0.63–0.70 respectively [46-47]. The higher the genetic diversity and PIC value reflects the population

contains the diverse accessions originated from different geographic location [35]. The SSRs used in the study represented di-, tri-, tetra- and penta- nucleotide repeat units. The allele sizes among the genotypes for all the SSRs varied from 106 to 255 bp. Number of repeats in the SSR motif had strong association with allele number and their polymorphism information content (PIC). SSRs having di-nucleotide repeats are the most polymorphic marker class followed by tri-, tetra- and penta-repeat units. The analysis of genetic diversity among the germplasm lines is of primary importance to plant breeders, as it contributes immensely to selection of parents, identification of QTLs based on association mapping study (allele mining) and prediction of potential genetic gains[35]. No much significant results were reported in the previous study based on the phenotypic data used to classify sorghum accessions. Limitations on use of morphological traits, their experimental costs, effect of environment, evaluation time and genotype × environment interactions are widely discussed [48-49]. Previous studies[8,50] reported a low but significant correlation ($r = 0.45$, $p < 0.01$) between molecular and phenotypic diversity classes using sorghum lines and concluded that the clusters of accessions formed based on the SSR markers data did not coincide with the clusters based on the phenotypic data, suggesting that the use of molecular markers for diversity analysis provided better results.

In this context, DNA-based SSR markers have become powerful application tools for illustrating and quantifying genetic differences within and among the various crop species. Microsatellite or SSRs have become markers of choice for studying genetic diversity in many crops owing to their multi-allelic nature, co-dominance, simplicity of use and reproducibility. With the availability of complete sorghum genomic sequence [51] on various online databases, thousands of genome-wide SSRs have been published in sorghum and have been employed in genetic diversity and other studies. All the markers used in the current study were polymorphic and gave multiple alleles in the range from 3 to 5. Genic SSRs have been reported to be less polymorphic compared with genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions [52-53]. The SSRs used in the study represented di-, tri-, tetra- and penta-nucleotide repeat units.

The present study analyze the pattern of divergence exists in a population of 20 sorghum accessions that constitute sorghum diversity panel for various studies. The average number of alleles per locus and gene diversity has indicated the existence of broad genetic base in this collection.

To our knowledge, this is the first report on the genetic relationship among Marathwada region cultivated sorghum lines based on microsatellite or SSR markers. Marathwada cultivated sorghum that included lines from various districts of Marathwada region showed a wide genetic background, but lines from the same origin tended to have a close genetic background.

Conclusion

The SSR markers used in the present study demonstrated the utility in diversity analysis among the set of sorghum accessions. The genetic diversity analysis represents the genetic diversity of germplasm accessions which could be helpful of broadening the base of breeding program. A Neighbor-joining tree was constructed (Nei 1973) [31] and showed clustering of genotypes into three groups. This indicated the genetic distinctness of germplasm collection of Marathwada region. The mean major allele frequency, gene diversity and polymorphic information content (PIC) was recorded and found to be 0.51, 0.61 and 0.55 respectively. This reflects the broad genetic variability was existed across the germplasm accessions that provided the scope to identify the allelic variants (superior alleles) through candidate gene-based association mapping approach in rice breeding program.

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