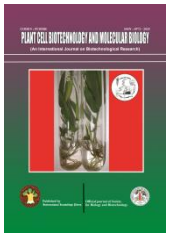

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# Evaluation of Genetic Diversity in Sorghum Genotypes via Simple Sequence Repeat (SSR) Markers

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author NR conducted the research as part of her Ph.D. thesis and wrote the manuscript. Author MUV was involved in finalizing the research topic, facilitating the research work, provided technical guidance, and reviewed the draft. Authors NP, BB and HNG worked as Advisory Committee members, provided technical guidance for the research and contributed to the review and correction of the drafts. All authors read and approved the final manuscript.*

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## ABSTRACT

This study aimed to evaluate the genetic diversity of 20 sorghum [Sorghum bicolor (L.) Moench] genotypes *via* simple sequence repeat (SSR) markers, with a focus on identifying drought-tolerant varieties. Leaf samples were collected from three-week-old seedlings, and genomic DNA was

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extracted *via* the CTAB method, a widely recognized protocol for obtaining high-quality DNA. The DNA samples were quantified to ensure consistent template concentrations for amplification *via* 25 SSR primers. PCR amplification was performed, followed by agarose gel electrophoresis to separate and visualize the SSR band patterns, which were then recorded in a binary matrix format on the basis of the presence or absence of bands. Among the SSR primers used, nine were polymorphic, generating 13 scorable markers, highlighting the genetic variability among the genotypes. The polymorphic information content (PIC) values ranged across the SSR loci, with the Xtxp145 locus exhibiting the highest PIC value of 0.998, indicating its high discriminatory power and informativeness in differentiating between genotypes. Genetic similarity indices were calculated *via* Jaccard's similarity coefficient [1], and the data were subjected to cluster analysis *via* the unweighted pair group method with arithmetic mean (UPGMA) method. The resulting dendrogram grouped the genotypes into seven main clusters at a 50% similarity threshold, underscoring the genetic diversity present within the sorghum genotypes studied. Cluster I contained a single genotype, SVD-1272R, whereas, Cluster II included seven genotypes with subcluster formation. Cluster III comprised one ungrouped genotype, SPV-486. Cluster IV included eight genotypes, whereas Clusters V, VI, and VII each contained a single ungrouped genotype. This dendrogram illustrates the genetic diversity and relationships among the sorghum genotypes on the basis of similarity indices. The findings of this study confirmed the efficacy of SSR markers in assessing genetic diversity and emphasized their potential utility in breeding programs aimed at improving drought tolerance.

*Keywords: Sorghum; SSR; PIC; Primers; similarity indices.*

## 1. INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], known as the “King of Millets” for its large grain size, is a key cereal crop grown in both the kharif (rainy) and rabi (postrainy) seasons. In India, Maharashtra, Karnataka, and Andhra Pradesh are the major sorghum-producing states, contributing 80% of the national output, which constitutes approximately 16% of the global production. Despite only 5% of the sorghum area being irrigated, rabi sorghum is crucial for rainfed regions in Peninsular India. Drought stress negatively affects sorghum's physiological and biochemical processes, necessitating improvements in drought tolerance traits such as root growth, leaf development, and water use efficiency [2]. However, phenotypic selection for these traits is complex because of the interaction of degree of stress during grain filling and panicle sink size [3]. To address these challenges, evaluating sorghum genotypes for genetic diversity [4] and drought tolerance is essential [5]. Genetic diversity within cultivated species is a valuable resource for enhancing crop productivity and quality and for developing pest-resistant varieties. Molecular markers, particularly simple sequence repeats (SSRs), offer a robust approach for assessing genetic variation and identifying drought-resistant genotypes [6]. SSR markers, owing to their high reproducibility and multiallelic variation, are valuable for genomic mapping and marker-assisted selection [7]. This study utilized SSR markers to explore genetic diversity among sorghum genotypes, aiming to enhance breeding programs and develop high-yielding, drought-tolerant varieties [8]. This research was needed to identify genetic diversity among sorghum genotypes, which is crucial for breeding programs focused on enhancing drought tolerance. Understanding this diversity allows for the selection of robust genotypes that can withstand water scarcity. Additionally, the use of SSR markers provides precise insights into the genetic relationships among the genotypes, aiding in the efficient development of improved sorghum varieties. This investigation aimed to identify the genetic diversity of sorghum genotypes for drought tolerance *via* SSR markers.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Leaf Material and Isolation of DNA

In this study, twenty rabi sorghum genotypes were obtained from AICRP-Sorghum, College of Agriculture, Dharwad. Seeds were sown in pots within the Crop Physiology Department laboratory to isolate genomic DNA. Leaf samples were collected from 15-20 days old seedlings early in the morning. The CTAB method was employed for DNA isolation, and 2% CTAB detergent and high NaCl concentrations were

used to facilitate cell lysis. This process breaks down the cell walls, membranes, and nuclear membranes, with CTAB precipitating polysaccharides and chloroform denaturing and precipitating proteins. DNA was then precipitated using ice-cold isopropanol [9]. For extraction, 200 mg of each leaf sample was ground in liquid nitrogen and transferred to centrifuge tubes containing prewarmed extraction buffer. The mixture was incubated at 65°C for 45 minutes, then centrifuged at 13,000 rpm for 10 minutes. The supernatant was treated with phenol:chloroform:isoamyl alcohol (25:24:1) mixture and centrifuged again. The supernatant was then treated with a chloroform:isoamyl alcohol (24:1) mixture and centrifuged. The DNA-containing aqueous phase was precipitated with chilled isopropanol and incubated at 4 ºC overnight. After centrifugation, the DNA pellet was washed, air-dried, and resuspended in T10E1 buffer. RNase was added, and the mixture was incubated at 37 ºC to remove RNA. The final DNA samples were stored at -20 ºC for further analysis.

## 2.2 PCR Amplification and Optimization of the DNA Concentration for PCR

PCR amplification was performed in a 10 µl reaction mixture using specific primers (Table 1), with the products stored at 4°C until gel electrophoresis. DNA concentrations were optimized to 50 ng/µl *via* TE buffer, on the basis of electrophoresis intensity.

## 2.3 Primers and Optimization of the Annealing Temperature for PCR Amplification

For PCR amplification, 25 SSR primers were used (Table 3). The annealing temperatures of some primers were determined *via* gradient PCR amplification (Table 2), with the PCR program run in a thermal cycler.

## 2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted *via* a 3% agarose gel prepared in 1.0X TAE buffer with 0.5 µg/ml ethidium bromide. After solidifying, the gel was transferred to a tank with 1.0X TAE buffer and loaded with PCR products. Electrophoresis was run at 100 V until the dye migrated two-thirds of the distance. The gel was visualized under UV light at 300 nm and documented *via* the ALPHA IMAGER Gel Documentation Unit.

## 2.5 SSR Analysis

The distinctly separated PCR-amplified bands of various sorghum genotypes obtained *via* different SSR primers (Table 3) were manually recorded in a binary matrix format on the datasheet, with bands marked as present (1) or absent (0).

**Table 1. Contents of the PCR mixture**

| Reaction components | Concentration | Volume (µl) |
|---|---|---|
| Sterile distilled water | - | 3.5 |
| Genomic DNA | 30-50 ng/µl | 0.5 |
| Forward primer | 10 pico moles | 0.5 |
| Reverse primer | 10 pico moles | 0.5 |
| Master mix (dNTPs, Taq DNA polymerase, $MgCl_2$, buffer) | - | 5.0 |
| Total | | 10 |

**Table 2. Program for PCR amplification**

| Steps | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | |
| Denaturation | 95 | 30 sec | |
| Annealing | 60 | 30 sec | |
| Extension | 72 | 90 sec | 36 |
| Final extension | 72 | 10 min | |
| Hold | 4 | Hold | |

**Table 3. List of SSR markers**

| Sl. No. | Name | Sequence | Traits | Reference |
|---|---|---|---|---|
| 1 | Xtxp335 | F- TATTTCCTCTTGAAAGAATCAGGG<br>R- TATTCATCGAGCAAAAGGCA | Culm diameter | |
| 2 | Xtxp149 | F- AGCCTTGCATGATGTTCC<br>R- GCTATGCTTGGTGTGGG | Leaf dry weight | Edema and Amoding [12] |
| 3 | PepC | F- TGGGAAGCAGCTCAGG<br>R- AGGGTGGTGATGTAGGGA | Panicle exertion | |
| 4 | Xtxp315 | F- AACCTACCCTAGCCCGCGCAACTG<br>R- CAACATGTCCGCAAGATTTGATGTGAC | Panicle length | |
| 5 | Xtxp32 | F- AGAAATTCACCATGCTGCAG<br>R- ACCTCACAGGCCATGTCG | Flag leaf length | Edema and Amoding [12] |
| 6 | Xtxp 145 | F- GTTCCTCCTGCCATTACT<br>R- CTTCCGCACATCCAC | Number of leaves, Root dry weight, root length and stem diameter | Ram *et al*. [13] |
| 7 | Xtxp320 | F- TAAACTAGACCATATACTGCCATGATAA<br>R- GTGCAAATAAGGGCTAGAGTGTT | Number of leaves and panicle length | Ram *et al*. [13] |
| 8 | Xtxp30 | F- AAAAAGGACGCGCAGCTG<br>R- CTGGTCTCCACCATCCGTAG | Panicle length | |
| 9 | Xtxp197 | F- GCGTCAATTAATCCAAACAGCCTC<br>R- GAGTTCCTATTCCCGTTCATGGTGAT | Seed yield / plant | |
| 10 | Xtxp51 | F- TCTCGGACTCAAGAGCAGAGG<br>R- GGACAGCAGCGGCTTCAG | Root Volume | Edema and Amoding [12] |
| 11 | Xtxp270 | F- AGCAAGAAGAAGGCAAGAAGAAGG<br>R- GCGAAATTATTTTGAAATGGAGTTGA | Root Volume and Panicle no./plant | |
| 12 | X cup 63 | F- GTAAAGGGCAAGGCAACAAG<br>R- GCCCTACAAAATCTGCAAGC | Number of fully expanded leaves | Edema and Amoding [12] |
| 13 | Xtxp136 | F- GCGAATAGCATCTTACAACA<br>R- ACTGATCATTGGCAGGAC | Root length | |
| 14 | Xtxp265 | F- GTCTACAGGCGTGCAAATAAAA<br>R- TTACCATGCTACCCCTAAAAGTGG | Shoot and root fresh weight | Edema and Amoding [12] |
| 15 | Xtxp273 | F- GTACCCATTTAAATTGTTTGCAGTAG<br>R- CAGAGGAGGAGGAAGAGAGG | Shoot and root dry weight | |
| 16 | Xtxp021 | F- GAGCTGCCATAGATTTGGTCG<br>R- ACCTCGTCCCACCTTTGTTG | Root to shoot ratio | Ram *et al*. [14] |

| Sl. No. | Name | Sequence | Traits | Reference |
|---|---|---|---|---|
| 17 | X cup02 | F- GACGCAGCTTTGCTCCTATC<br>R- GTCCAACCAACCCACGTATC | Average leaf area and root to shoot ratio | Mofokeng *et al*. [14] |
| 18 | mSbCIR300 | F- TTGAGAGCGGCGAGGTAA<br>R- AAAAGCCCAAGTCTCAGTGCTA | Shoot length and shoot diameter | Mofokeng *et al*. [14] |
| 19 | Xtxp88 | F-CGTGAATCAGCGAGTGTTGC<br>R-TGCGTAATGTTCCTGCTC | Shoot and root dry weight | Mofokeng *et al*. [14] |
| 20 | X Gap 72 | F- TGCCACCACTCTGGAAAAGGCTA<br>R- CTGAGGACTGCCCCAAATGTAGG | Shoot length, stem diameter | Edema and Amoding [12] |
| 21 | X Cup 53 | F- GCAGGAGTATAGGCAGAGGC<br>R- CGACATGACAAGCTCAAACG | Number of fully expanded leaves | Edema and Amoding, [12] |
| 22 | mSbCIR262 | F- GCACCAAAATCAGCGTCT<br>R- CCATTTACCCGTGGATTAGT | Shoot length, stem diameter and no. of fully expanded leaves | Mofokeng *et al*. [14] |
| 23 | MSbCIR306 | F- ATACTCTCGTACTCGGCTCA<br>R- GCCACTCTTTACTTTTCTTCTG | Shoot length | Mofokeng *et al*. [14] |
| 24 | X Gap 206 | F- ATTCATCATCCTCATCCTCGTAGAA<br>R- AAAAACCAACCCGACCCACTC | Shoot fresh weight | Mofokeng *et al*. [14] |
| 25 | X cup 03 | F- ATCCAACCAACCCACGTATC<br>R –TACGCAGCTTTGCTCCTATC | Average leaf area | Edema and Amoding [12] |

### 2.6 Statistical Analysis for Construction of Dendrograms

A data matrix was created from band scores, with similarity index (SI) values computed as the proportion of shared bands between genotypes. Using NTSYS-PC version 2.02 and the unweighted pair group method with arithmetic mean (UPGMA), a dendrogram was generated on the basis of Jaccard's similarity coefficient [1]. Binary data were analysed with the Dice similarity coefficient via the SIMQUAL module, and the SAHN module was used for dendrogram construction *via* the UPGMA method [10].

### 2.7 Polymorphic Information Content (PIC)

For the assessment of marker informativeness, the PIC of each SSR was computed *via* the formula: PIC = 1 - $\sum pi^2$, where pi represents the frequency of the $i^{th}$ allele [11]. The Polymorphism Information Content serves as a measure of the discriminatory ability of an SSR marker locus.

## 3. RESULTS AND DISCUSSION

### 3.1 SSR Analysis

Genetic diversity among 20 sorghum genotypes was analysed *via* 25 SSR markers, with 9 primers identified as polymorphic, resulting in the identification of a total of 13 scorable markers. The number of markers per primer ranged from two to four, with primer Xtxp315 generating the most (four products) and Xtxp335 the fewest (two products). The polymorphic information content (PIC) values, which indicate the discriminatory power of each locus, vary among primers, reflecting the genetic variability within the genotypes [15]. SSR banding patterns for specific primers, including Pep C, Xtxp315, Xcup63, mSbCIR300, Xtxp265, and Xtxp88, are illustrated in Fig. 1.

### 3.2 Polymorphic Information Content

In this study, the polymorphic information content (PIC) values of SSR markers were used to assess their informativeness in evaluating genetic diversity among sorghum genotypes. Markers with PIC values of 0.5 or higher were considered informative. The PIC values varied across the SSR loci, with Xtxp145 showing the highest value of 0.998, indicating its high informativeness, whereas, Xtxp335 had the lowest value of 0.023, reflecting lower informativeness (Table 4). Xtxp 315 showed the polymorphic bands with 0.062 PIC value (Fig. 1). These findings align with the PIC ranges reported by Mofokeng *et al*. [14] suggesting that Xtxp145 is particularly valuable for diversity analyses because of its ability to detect a wide range of alleles.

### 3.3 Similarity Index

This study demonstrated the effectiveness of SSR markers in evaluating genetic diversity among sorghum genotypes and highlighted their utility in selecting optimal crosses and categorizing genotypes. Similarity indices, computed *via* NTsys software, provide insights into genetic relationships and similarities among genotypes [16]. These indices quantify genetic similarity on the basis of SSR marker profiles. The Jaccard similarity indices, which were calculated from the binary data of the polymorphic primers, ranged from 0.76 - 1 (Fig. 2). Genotype pairs such as G2 with G4 and G7-G11 with G12 and G13- G19 with G2-G4, and G7- G17 and G18 exhibited the highest similarity index of 1. Conversely, G3, with G5 and G20, had the lowest similarity index of 0.76.

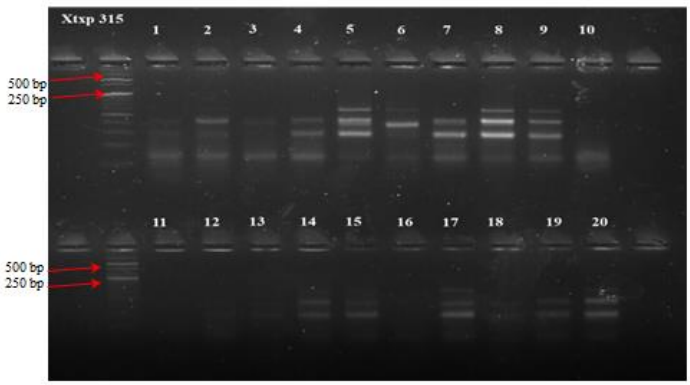


**Fig. 1. Images of an agarose gel showing the PCR amplification of the primers Xtxp 315**

**Table 4. Polymorphic information content (PIC) values generated through SSR primers among sorghum genotypes**

| Sl. No. | SSR markers | Annealing temperature (◦C) | PIC |
|---|---|---|---|
| 1 | Xtxp335 | 60 | 0.023 |
| 2 | Xtxp149 | 55 | 0.000 |
| 3 | PepC | 60 | 0.098 |
| 4 | Xtxp315 | 55 | 0.062 |
| 5 | Xtxp32 | 60 | 0.098 |
| 6 | Xtxp 145 | 60 | 0.998 |
| 7 | Xtxp320 | 54 | 0.190 |
| 8 | Xtxp30 | 60 | 0.000 |
| 9 | Xtxp197 | 60 | 0.000 |
| 10 | Xtxp51 | 60 | 0.000 |
| 11 | Xtxp270 | 60 | 0.000 |
| 12 | X cup 63 | 54 | 0.000 |
| 13 | Xtxp136 | 55 | 0.190 |
| 14 | Xtxp265 | 55 | 0.000 |
| 15 | Xtxp273 | 60 | 0.000 |
| 16 | Xtxp021 | 60 | 0.000 |
| 17 | X cup02 | 54 | 0.000 |
| 18 | mSbCIR300 | 61.2 | 0.000 |
| 19 | Xtxp88 | 54.5 | 0.000 |
| 20 | X Gap 72 | 60 | 0.098 |
| 21 | X Cup 53 | 56 | 0.000 |
| 22 | mSbCIR262 | 52.7 | 0.000 |
| 23 | MSbCIR306 | 56.4 | 0.098 |
| 24 | X Gap 206 | 56.6 | 0.000 |
| 25 | X cup 03 | 56 | 0.000 |

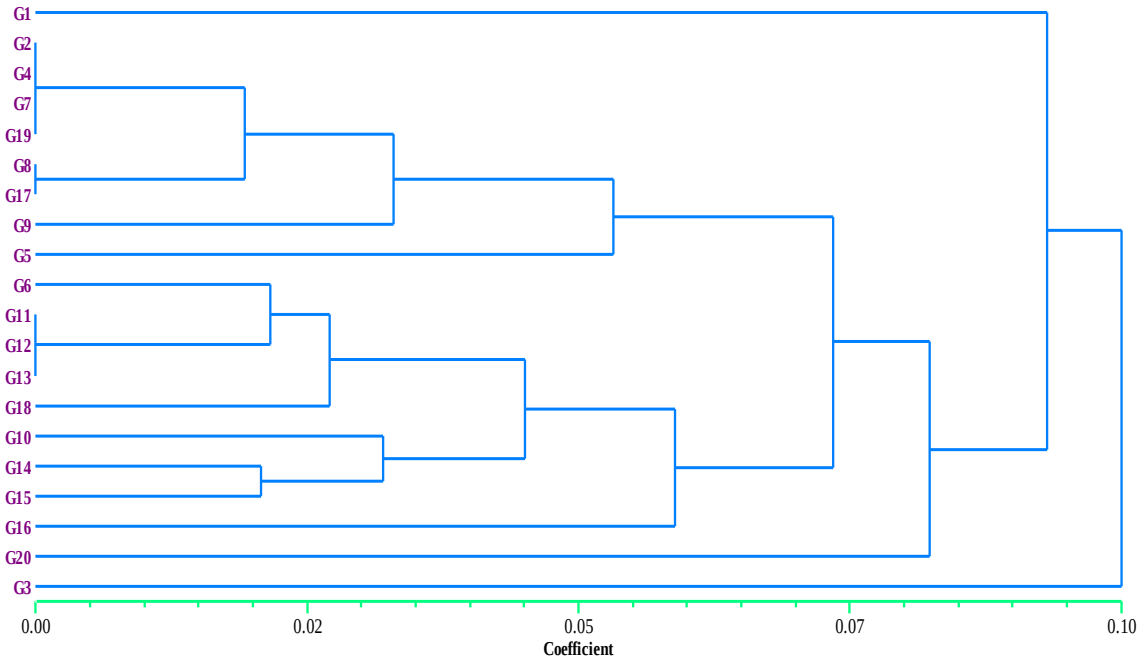


**Fig. 2. UPGMA-dendrogram on the basis of jaccard's similarity coefficient Cluster analysis based on genotyping of accessions with SSR markers**

**Table 5. Distribution of *rabi* sorghum genotypes into different clusters on basis of SSR data**

| Cluster (at 0.05 coefficient) | Sub cluster (at 0.02 coefficient) | No. of genotypes | Genotypes name |
|---|---|---|---|
| Cluster I | | 1 | G1 (SVD-1272R) |
| Cluster II | Cluster II a | 6 | G2 (SVD-1358R), G4(SVD-1403R), G7(CSV-216R),G19 (BJV-44), G8 (CSV-29R), G17 (Basavan moti) |
| | Cluster II b | 1 | G9 (ICSR-15001) |
| Cluster III | | 1 | G5 (SPV-486) |
| Cluster IV | Cluster IV a | 4 | G6 (SPV-2217), G11(TandurL), G12(Phule Anuradha), G13(Chitapur - L) |
| | Cluster IV b | 1 | G18 (Phule Vasudha) |
| | Cluster IV c | 1 | G10 (Basavana pada) |
| | Cluster IV d | 2 | G14 (DKS- 35), G15 (M-35-1) |
| Cluster V | | 1 | G16 (M 148-138) |
| Cluster VI | | 1 | G20 (ICSR- 13025) |
| Cluster VII | | 1 | G3 (SVD-1528R) |

Similarity indices are crucial for clustering genotypes with similar genetic profiles, aiding in the grouping of genotypes into distinct clusters [17]. Clustering algorithms, such as hierarchical clustering, help reveal population structure and genetic relationships [4]. These indices are key in constructing dendrograms or phylogenetic trees, which illustrate genetic diversity [18]. In this study, 20 sorghum genotypes were clustered *via* SSR markers, resulting in seven main clusters at a 50% similarity threshold (Table 5). Cluster I contained a single genotype, SVD-1272R. Cluster II included seven genotypes with subcluster formation. Cluster III had one ungrouped genotype, SPV-486. Cluster IV comprised eight genotypes, whereas Clusters V, VI, and VII each contained a single ungrouped genotype. This dendrogram illustrates the genetic diversity and relationships among the sorghum genotypes on the basis of similarity indices.

## 4. CONCLUSION

This study evaluated the genetic diversity of 20 sorghum genotypes *via* SSR markers to identify drought tolerance traits. Among the 25 primers, nine were polymorphic, generating 13 scorable markers with various polymorphic information content (PIC) values. The genotypes were categorized into seven clusters on basis of similarity indices, revealing distinct genetic relationships. Notably, genotypes G2, G4, G17, G19 and G7 presented the highest similarity indices, suggesting potential drought tolerance traits. This genetic diversity evaluation is critical for selecting genotypes for breeding programs aimed at enhancing drought tolerance and overall crop productivity in sorghum.

### DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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