SSR Marker-based DNA fingerprinting and morphological characterization for varietal identification in popular sorghum varieties of Tamil Nadu

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Abstract
DNA fingerprinting of varieties is mandatory for registration of germplasm and notification of newly released varieties. The present study attempted to develop a DNA fingerprinting profile of newly released varieties of sorghum using publically available SSR markers along with morphological DUS descriptors. Twenty-one SSR markers were used for the identification of unique variety-specific fingerprints in nine varieties/cultures. Of them, 14 primers (66.7%) showed clear and unambiguous amplification which is good enough to identify unique banding patterns for specific cultivars (77.8%). The SSR markers Xtxp024, Xtxp231, Xtxp075 produced unique alleles in CO 32 whereas Xtxp354 produced an unique alleles in K12. The SSR marker Xtxp003, Xtxp201 produced unique alleles in CSV 33 MF which could serve as valid genotype-specific SSR markers in varietal purity test program. The varietal-specific SSR marker will supplement the DUS test and could play a major role in varietal identification, thus resolving disputes during the seed certification process.

Keywords
DUS test, SSR fingerprinting, varietal identification and protection

INTRODUCTION
Sorghum (Sorghum bicolor (L.) Moench), an annual diploid (2n=2x=20) ranks fifth among global cereal crops next to maize, rice, wheat and barley (FAOSTAT 2015) with a global production of about 57.10 million metric tonnes (USDA, 2019). More than half of world sorghum production is from the semi-arid regions of Africa and Asia and one of the dietary staples of the worlds poorest, especially in the semi-arid tropics. It is notable for its multiple economic uses like food, fodder, biofuel, and other industrial uses.

Elite cultivars coupled with High-quality seeds play an indispensable role in the production. Varietal identification is of prime importance worldwide from the perspective of Plant Variety Protection (PVP). Protection can be granted if the newly evolved cultivar satisfies Distinctiveness, Uniformity, and Stability (DUS) (Prajapati et al. 2018). Since new cultivars were developed through hybridization between members of elite groups of genetically similar parents, the genetic variability among newly evolved cultivars were found to be even smaller (Rahman et al., 2009). This makes the unambiguous distinction of cultivars to be difficult from the rest based on DUS tests which consist of morphological and physiological characteristics. For varietal registration under “Protection of Plant Varieties and Farmer’s Rights (PPV&FR) Act, 2001” the newly evolved cultivar must fulfill the DUS test. Elite sorghum genotypes were first selected based on morphological characterization (Beta and Corke, 2001). However, from several studies, it is significant...
that morphological markers alone are insufficient due to low level of heritability, low level of abundance and is highly influenced by the environment (Seetharam and Ganesamurthy, 2013, Verma et al. 2017, Bhusal et al. 2017, Prajapati et al. 2018).

However, Molecular markers based on fingerprinting allows precise, objective and rapid cultivar identification that are challenging to characterize due to the identical morphological characters or indistinct traits (Galovic et al., 2006). It’s clear from several studies that several types of molecular markers such as RAPD (Sorghum: Mehmoon et al., 2008), AFLP (Wheat: Heun et al., 1997) and SSR marker (Sorghum: Bantte and Mogus, 2016) can be used for genetic fingerprinting.

However, the Simple Sequence Repeat (SSR) represents a high degree of polymorphism, reproducibility, co-dominance and multi allelic types of variation (Becher et al., 2000) and hence widely used in genetic analysis and cultivar identification. Applicability of SSR markers in cultivar identification has been reported in Rice (Rahman et al., 2009), Grapes (Dangl et al., 2001) and Potato (Coombs et al., 2004).

In recent years, fingerprinting the commercial sorghum cultivars based on molecular markers is of paramount significance for unambiguous and quick identification of similar or closely related varieties which could prevent the disputes arising due to varietal ownership. The applicability of SSR markers in Sorghum Fingerprinting has been reported by Bantte and Mogus, 2016 and Gangurde et al., 2016. The DNA fingerprinting is developed for the released sorghum varieties of Ethiopia (Bantte and Mogus, 2016). Therefore, this study intended to probe the appliance of the molecular marker in the context of DUS tests to disclose unique variety-specific fingerprints. This varietal fingerprint could be used for various varietal purity test programs of closely related sorghum cultivars and submission of fingerprint data for the crop variety registration.

**MATERIALS AND METHODS**

A total of eight elite sorghum cultivars were studied. These genotypes were chosen based on their significant role in the seed production system and are categorized based on their use in agricultural practices: grain sorghum (K8, K12, CO(S) 28, CO 30, CO 32), fodder sorghum (CSV 33MF) and pre-release cultures which are in the advanced stage of yield trials like MLT and ART (TNS 660, TNS 661) (Table 1). The popular sorghum varieties along with cultures were evaluated for various agro-morphological traits based on 31 DUS descriptors provided by Protection of Plant Variety and Farmers Right Act (PPV&FRA, 2007).

A set of twenty-one informative SSR markers were used for identification of unique variety-specific fingerprint. These SSR markers were picked based on their maximum genome coverage. The SSR markers include Xtxp003, Xtxp024, Xtxp027, Xtxp030, Xtxp031, Xtxp038, Xtxp043, Xtxp051, Xtxp058, Xtxp075, Xtxp088, Xtxp145, Xtxp201, Xtxp231, Xtxp274, Xtxp285, Xtxp286, Xtxp287, Xtxp297, Xtxp312 and Xtxp354. The SSR markers PCR conditions validated by Shehzad et al. 2008, Kong et al., 1999 is used in the present study (Table 2).

**Table 1. List of sorghum genotypes used in this study**

| Varieties/ cultures | Pedigree | Year of release | Special characters |
|---------------------|----------|-----------------|--------------------|
| K8                  | IS 12611 × SC 108 | 1989 | Rainfed |
| K12                 | SPV 772 x S 35–29 | 2014 | Dual-purpose variety |
| CO(S) 28            | CO 25 × SPV 942 | 2001 | High ylde, short duration, non-lodging, resistant to shoot borer |
| CO 30               | APK 1 × TNS 291 | 2010 | High dry matter digestibility, moderately resistant to shoot fly, resistant to downy mildew |
| CO 32               | APK 1 × M 35-1 | 2020 | Dual-purpose variety, high protein content, moderately resistant to shoot fly and stem borer |
| TNS 660             | TNS 603 × EP 60 | * | Short duration |
| TNS 661             | TNS 603 × IS 18551 | * | Moderately resistant to shoot fly |
| CSV 33 MF EMS Mutant of COFS 29 | 2016 | Forage sorghum, tall, thin stem, high tillering |

*Pre-release cultures

Seedlings were raised under the greenhouse during November 2018. Genomic DNA from each cultivar was obtained from fresh leaf tissues of two-week-old seedlings based on the modified CTAB method (Grewal et al., 2013). DNA quality and quantity were obtained photometrically by Bio-Spectrometer, Kinetic (Eppendorf, Germany) and visually by agarose gel electrophoresis (0.8% agarose gel).

PCR amplification was performed in Eppendorf, Mastercycler Gradient, Germany. Polymerase Chain Reaction (PCR) was set out for 10µl comprising master mix (smART Prime) 7 µl, Forward primer 0.5 µl, reverse primer 0.5 µl, DNA 1 µl, and water 1 µl. The amplification profile comprised of Initial denaturation of template DNA at 94°C for 5 mins and subsequent 35 cycles each with Denaturation at 94°C for 1 min, Annealing at 55°C to 60°C.
For 1 min. Extension at 72°C for 1 min. In the last cycle, the final extension was provided at 72°C for 7 min. For the separation of PCR product, electrophoresis was carried out on a 3% agarose gel containing Ethidium bromide using 1X TBE buffer (pH 8.0). The amplified products were visualized under UV light source (Bio-Rad, CA and USA). Only clear and unambiguous SSR alleles were scored based on base pair (bp) size in each genotype.

**Table 2. List of SSR markers, Primer Sequence, Repeat Motif, and Annealing Temperature**

| Marker name | Chromosome no. | Repeat motif | Forward primer | Reverse primer | Annealing Temperature |
|-------------|----------------|--------------|----------------|-----------------|-----------------------|
| Xtxp003     | 2              | (CT)$_3$+(CT)$_3$ | AGCACGCCTTTATGGAAG | ATCCCTCATACTGCAGGACC | 50 200-235          |
| Xtxp024     | 4              | (TC)$_{21}$ | TTGTGTAGTCATCCAGTGC | TTCTAAAGCCACCCAGATTTG | 60 145-160          |
| Xtxp027     | 4              | (AG)$_{7}$ | AATTTTTGCTTTATGGAAG | GCGCAGGCTTTATGGAAG | 45 332              |
| Xtxp030     | 10             | (AAT)$_{25}$ | AAAAAAGACAGCAAGCTG | CTGGTACTCCACCATCCGTAG | 60 290-300          |
| Xtxp031     | 3              | (CT)$_{25}$ | TGCAGGCCTGGCTCTAG | TTCTCAATCTATTGGAAG | 60 222              |
| Xtxp038     | 3              | (AG)$_{17}$ | CAAACCCGACGAGTGAAC | ACAAGGCAAGCACAAGAG | 60 437              |
| Xtxp043     | 1              | (CT)$_{28}$ | AGTCAAGACACACTGCTCTG | AATTACCTGGCCGCTCTGC | 60 170              |
| Xtxp051     | 4              | (TG)$_{11}$ | TCTCGGACTCAAGAGGAGG | GGACAGCAGCCGCTTCAG | 60 225-230          |
| Xtxp058     | 1              | (AG)$_{15}$+(GA)$_{16}$ | TTTTTGCTTTATGGAAG | TTCCCTTGTGCTGGTTTGGT | 55 145-160          |
| Xtxp75      | 1              | (TG)$_{10}$ | CGATGCCTGAAAAAAAACG | CGATCAGAGGGTGAGGAGG | 50 140-170          |
| Xtxp088     | 1              | (AG)$_{31}$ | CTGTAAATCGAGGATGTGG | TGCAGAATGCTTCCTC | 53 150-190          |
| Xtxp145     | 1              | (AG)$_{22}$ | GTTCTCCTTGGCATTACT | CTTCCTCGACTCCAC | 60 200-230          |
| Xtxp201     | 2              | (GA)$_{38}$ | GCGTTATGAGAAGAAAT | CTCAAAAGCCAGGGAGAC | 60 225-265          |
| Xtxp231     | 3              | - | GAAATACAGGAGTAGGT | AGGCAAGGCTGATCA | 55 150-178          |
| Xtxp274     | 9              | (TTC)$_{19}$ | GAAATATACTGGCTACCCCTAAAAGT | ACTCTACTCCCGCTCCACAT | 60 280-320         |
| Xtxp285     | 3              | (CT)$_{17}$ TCT(CT)$_{19}$ | ATTTGATTCTCTTCTGTTTGGCC TTGT | TTGCATTCTCCCTCTCTTCTTTTT | 60 205-260          |
| Xtxp286     | 2              | (GCA)$_{18}$ ACA (GCA)$_{10}$ | AGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAGCAAG | GCAGTGGCTCTTTGTGTTC | 55 190-220          |
| Xtxp287     | 6              | (AAC)$_{21}$ | GCAAGGAGCTGACTTTAATAGAAG | CAAAGTGTCTACTAAACCCCTATAGCAAGGGTAA | 60 330-360          |
| Xtxp297     | 2              | (AAG)$_{24}$ | GAACCATATGTGTTTAAGTGC | GCACAAATCTCCGCTAATCAACAT | 55 170-400          |
| Xtxp312     | 5              | (CAA)$_{20}$ | CAGGAAATACAGTCCAGTGCC AAGT | GTGAATAATCCGAAAGAGGAGTTT | 60 90-185           |
| Xtxp354     | 8              | (GA)$_{25}$+(AAG)$_{4}$ | TGGGCAGGCTATGCTCTAATGA | GCGCTTTTCTGAGGCTTTG | 60 130-170          |
RESULT AND DISCUSSION

Morphological markers have been frequently used in the genus Sorghum for descriptive purposes and are used in plant variety protection for distinguishing the individual varieties based on its distinctness, uniformity, and stability (DUS) test (Prajapati et al. 2018). Characterization and evaluation of the accessions are the pre-requisites for the utilization of the available diversity in breeding perspective. Hence, the sorghum varieties are characterized to identify cultivar specific traits (Table 3) and cultivar specific SSR fingerprint which could be used for variety identification.

Molecular fingerprinting is of utmost importance in protecting the novelty of a newly evolved plant variety. In the present study 21 SSR primer pairs were used to score a set of eight cultivars. Among them, 14 primers (66.7%) showed clear and unambiguous amplification, which is good enough to identify unique allelic patterns for specific cultivars (77.8%). Further, two sorghum cultivars K8 and TNS 661 could not be distinguished, that indicated their lower genetic variability possibly due to close relatedness and a limited number of polymorphic SSR markers. The cultivars K8, TNS 660 and CSV 33 MF could be easily differentiated during the early growth stages from other varieties based on greyed purple pigmentation in coleoptile as well as leaf sheath. The SSR marker Xtxp297 produced unique alleles in TNS 660 (200bp) and CSV 33 MF (190bp) and hence it can be used for differentiation of these two cultivars. On the other hand, Xtxp003 and Xtxp201 can be used for differentiating CSV 33 MF (205bp, 180bp) from TNS 660 (Fig. 1b). Hence the markers Xtxp003, Xtxp201 can be designated as genotype-specific SSR markers for identifying CSV 33 MF in varietal purity testing programs. Bhusal et al. (2017) reported the presence of greyed purple pigmentation on seedling could be correlated with tannin content in seeds. It is proven by various studies that the presence of purple pigmentation on leaf sheath had a positive correlation \( r = 0.56 \) with shoot fly dead hearts (%) (Mayilsamy et al. 2017).

Table 3. Cultivar specific distinguishing DUS traits of sorghum

| S.NO | TRAITS | SCORE* | K8 | K12 | CO | S | CO | S28 | CO | S30 | CO | S32 | TNS 660 | TNS 661 | CSV 33MF |
|------|--------|--------|----|----|----|---|----|-----|----|----|----|----|-----|--------|--------|--------|
| 1    | Seedling: Anthocyanin colouration of coleoptile | 1-2 | 2  | 1  | 1  | 1  | 1  | 2   | 1  | 1  | 2  | 2  |     |        |        |        |
| 2    | Leaf sheath: Anthocyanin colouration | 1-2 | 2  | 1  | 1  | 1  | 1  | 2   | 1  | 1  | 2  | 2  |     |        |        |        |
| 3    | Plant: time of panicle emergence | 1-9 | 7  | 3  | 3  | 5  | 3  | 5   | 7  | 5  | 7  | 5  |     |        |        |        |
| 4    | Lemma: Arista formation | 1,5 | 1  | 1  | 1  | 1  | 1  | 1   | 1  | 1  | 1  | 5  |     |        |        |        |
| 5    | Stigma: anthocyanin colouration | 1,5 | 5  | 1  | 1  | 1  | 1  | 1   | 5  | 5  | 5  | 5  |     |        |        |        |
| 6    | Stigma: yellow colouration | 1,5 | 5  | 5  | 1  | 1  | 1  | 1   | 5  | 5  | 5  | 5  |     |        |        |        |
| 7    | Stigma: Length | 3-9 | 5  | 3  | 3  | 3  | 3  | 3   | 3  | 3  | 5  | 3  |     |        |        |        |
| 8    | Flower with pedicel: Length of flower | 1-9 | 7  | 5  | 5  | 5  | 5  | 5   | 5  | 5  | 9  | 5  |     |        |        |        |
| 9    | Anther: Length | 3-7 | 3  | 5  | 5  | 5  | 5  | 5   | 3  | 3  | 3  | 3  |     |        |        |        |
| 10   | Anther: Colour of dry anther | 1-4 | 4  | 2  | 2  | 2  | 2  | 2   | 3  | 4  | 4  | 4  |     |        |        |        |
| 11   | Glume: colour | 1-6 | 4  | 3  | 3  | 3  | 3  | 3   | 5  | 4  | 6  | 5  |     |        |        |        |
| 12   | Plant: Total height | 1-9 | 5  | 5  | 5  | 5  | 5  | 7   | 3  | 3  | 7  | 5  |     |        |        |        |
| 13   | Stem diameter | 3-7 | 5  | 3  | 3  | 3  | 3  | 3   | 5  | 5  | 3  | 3  |     |        |        |        |
| 14   | Leaf: Length of blade | 3-9 | 7  | 7  | 7  | 7  | 7   | 5  | 7  | 7  | 7  | 9  |     |        |        |        |
| 15   | Leaf: Width of blade | 3-9 | 9  | 7  | 7  | 7  | 5  | 7   | 5  | 7  | 7  | 3  |     |        |        |        |
| 16   | Panicle: Length without peduncle | 1-9 | 7  | 3  | 3  | 3  | 5  | 5   | 5  | 5  | 9  | 5  |     |        |        |        |
| 17   | Panicle: Length of branches | 3-9 | 7  | 5  | 5  | 5  | 5  | 3   | 5  | 5  | 9  | 5  |     |        |        |        |
| 18   | Panicle: Density at maturity | 1-9 | 5  | 7  | 7  | 7  | 7  | 7   | 7  | 7  | 1  | 9  |     |        |        |        |
| 19   | Panicle: Shape | 1-5 | 3  | 3  | 3  | 3  | 3  | 3   | 3  | 5  | 3  | 5  |     |        |        |        |
| 20   | Neck of panicle: Visible length above sheath | 1-9 | 1  | 9  | 3  | 9  | 9   | 3  | 3  | 7  | 7  |      |        |        |        |        |
| 21   | Glume: Length | 1-9 | 3  | 7  | 5  | 5  | 5   | 1  | 1  | 1  | 9  |     |        |        |        |        |
| 22   | Grain: Threshability | 1-7 | 5  | 1  | 1  | 1  | 1   | 1  | 1  | 1  | 7  |     |        |        |        |        |
| 23   | Caryopsis: Colour after threshing | 1-5 | 3  | 3  | 3  | 3  | 3   | 3  | 3  | 3  | 5  |     |        |        |        |        |
| 24   | Grain: Shape(in dorsal view) | 1-3 | 3  | 3  | 3  | 3  | 2   | 3  | 3  | 2  | 3  |     |        |        |        |        |
| 25   | Grain: Shape in profile view | 1-3 | 2  | 2  | 2  | 2  | 3   | 3  | 3  | 3  | 2  |     |        |        |        |        |
| 26   | Grain: Size of mark of germ | 1-9 | 5  | 5  | 5  | 5  | 5   | 5  | 5  | 5  | 5  |     |        |        |        |        |
| 27   | Grain: Texture of endosperm | 1-9 | 5  | 3  | 3  | 3  | 3   | 3  | 3  | 3  | 5  |     |        |        |        |        |
| 28   | Grain: Colour of vitreous albumen | 1-3 | 1  | 1  | 1  | 1  | 1   | 1  | 1  | 1  | 2  |     |        |        |        |        |

* - Based on Guidelines for the conduct of test for Distinctiveness, Uniformity, and Stability on Sorghum (Sorghum bicolor (L.) Moench), PPV & FRA. 2007
At the time of peak flowering, K8 can be uniquely differentiated from other varieties based on the presence of anthocyanin pigmentation and yellow coloration in stigma. The varieties, K8 and CSV 33 MF have medium stigma length while others have a short stigma. Meanwhile, Xtxp312 can be used for differentiating CSV 33 MF (145bp) from K8 (Fig. 1a). Based on anther length, K8, TNS 660, TNS 661 and CSV 33 MF can be categorized as short whereas K12, CO (S) 28, CO 30 and CO 32 as medium. The medium-sized anther type varieties were further differentiated using the SSR marker. The SSR marker Xtxp145 differentiated K12 (210bp) and CO 30 (220bp) from CO (S) 28 and CO 32 by the presence of unique alleles whereas, Xtxp354 and Xtxp024 can be used for further differentiation of K12 (210bp) and CO 32 (160bp). Henceforth the SSR markers Xtxp354 and Xtxp024 can be used as a genotype-specific marker in varietal purity testing program. Absence of lemma arista formation (awn) reduced the evapotranspiration rate (Ayana and Bekele, 1998) which is noticed in all the tested varieties except CSV 33 MF. The presence of awn in CSV 33 MF acts as a defensive mechanism as bird scarers.

At physiological maturity, the genotypes K8, TNS 660, TNS 661 could be differentiated based on medium stem diameter (2 - 4 cm) while other varieties have small stem girth (< 2 cm). The larger stem girth denotes their resistance to lodging through enhanced culm strength. Broader leaf varieties like K8, K12, CO (S) 28, TNS 661 and CO 32 could ultimately be a high yielder because of more photosynthetic area. The SSR markers Xtxp274 can be used further for differentiating CO (S) 28 (310bp) from K8, K12, TNS 661 and CO 32 whereas Xtxp075 can be used for differentiating CO 32 (175bp) from K8, K12, CO (S) 28 and TNS 661. The dwarf genotypes could be used for developing desired plant types whereas genotypes with increased plant height are prone to lodging. But, beneficial as fodder, biomass fuel and thatching (Bhusal et al. 2017). From this perspective TNS 660 and TNS 661 were short (76 - 150 cm) whereas, CO 32 and CSV 33 MF were long (226 - 300cm). The SSR marker Xtxp231 can be used for differentiating CO 32 (210bp) from CSV 33 MF.
Panicle shape, compactness and length are important characters in determining grain yield and could be used as morphological marker for quicker varietal identification. CSV 33 MF can be uniquely differentiated from other varieties based on panicle shape like pyramidal type, whereas other varieties have symmetric type (Fig. 2). To further differentiate symmetric types, Xtxp058 can be used for differentiating K12 (190bp). The SSR marker Xtxp030 can be used for differentiating CO (S) 28 (305bp), CO 30 (290bp) and CO 32 (290bp). The open panicles could perform better in high rainfall and humid areas by avoiding mould and ergot diseases (Singh et al., 1997).

Moreover, K8 possess semi-loose panicle and CSV 33MF has a very loose panicle and rest of genotypes K12, CO (S) 28, CO 30, TNS 660, TNS 661 and CO 32 have semi-compact panicles.

The fodder sorghum variety CSV 33 MF had a very long glume cover compared to all the other grain sorghum varieties in the present study. The grain sorghum exhibits lesser glume coverage and could be easily threshable, while fodder sorghum exhibits higher glume coverage indicating difficulty in threshing (Verma et al., 2017). Moreover, the cultivar TNS 660 had greyed red glume color and CSV 33 MF had greyed purple glume color. Likewise, K8 and TNS 661 had greyed orange glume. Darker Glume color was found to be associated with grain mould resistance (Audilakshmi et al., 1999) in many of the sorghum varieties tested. The corneous endosperm was found to be correlated with grain mould resistance (Jambunathan et al.,1992 and Mukuru, 1992). The genotypes K12, CO (S) 28, CO 30, TNS 660, TNS 661 and CO 32 grains had 75% corneous endosperm. Hence, the glume color and endosperm texture can be used as a morphological marker for selecting parents in resistance breeding program. In addition to this, CSV 33 MF can be easily differentiated from the rest based on seed characters like caryopsis color after threshing (greyed orange), size of mark of germ (small) and color of vitreous albumen (greyed orange).

The discriminating morphological and DUS criteria can be efficiently used for varietal identification and grouping of varieties/cultures as grain or forage type. The SSR marker profile of Xtxp24, Xtxp231, Xtxp075, Xtxp354, Xtxp003 and Xtxp201 can be used for identification of specific cultivar. The unique variety-specific fingerprint obtained from the study can be used for varietal registration under the PPV and FR Act for obtaining plant varietal protection. This will also be used in varietal identification for consumer protection and resolving disputes in seed certification.

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