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Varietal identification and fingerprinting of Pearl Millet (*Pennisetum glaucum* L.) varieties and hybrid using morphological descriptors and SSR markers

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ABSTRACT

Pearl Millet (*Pennisetum glaucum*) is the sixth most important cereal crop in the world. The genomic resources available in Pearl millet can be utilized for fingerprinting and screening of hybrids using SSR markers and will be helpful for the assessment of seed purity. Hence, the present study was focused on fingerprint popular pearl millet varieties and hybrids of Tamil Nadu for varietal identification and hybrid purity test. The varieties used for DNA fingerprinting were CO (Cu) 9, CO 10, Pearl Millet hybrid CO 9 along with the parents, A' line ICMA 93111A and R' line PT 6029-30. The morphological features were recorded to screen the cultivars. The Pearl millet hybrid CO 9 scored the highest value for more than four quantitative characters via., Number of productive tillers (4-6), Leaf blade length (60-68cm), Leaf blade width (4.0-4.5cm), number of nodes (8-10), and 1000 seed weight (13-14g) which is at par and comparable with the composite CO 10 and higher than that of the variety CO (Cu) 9. PCR was performed using 36 SSR primers to find out polymorphism among the varieties. The SSR markers ICMP3021 and PSMP2089 were able to selectively identify CO (Cu) 9 from the other varieties. Whereas, the SSR markers ICMP3018, PSMP2219, and PSMP2220 were used to distinguish CO 10 from the other varieties. Further, the CO10 variety produced additional alleles for all the markers due to its composite nature. Among the thirty-six SSR primers screened, none of them were found suitable to distinguish the TNAU hybrid CO 9 from its parents. The unique DNA fingerprints developed in the present study can be utilized for seed purity testing and varietal identification.

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INTRODUCTION

Pearl millet is one of the sixth most and economically significant small seeded millet crop in the world. It contributes to 50% of world millet production. Pearl millet has its origin in Sahel of West Africa, where it was domesticated about 3000 years BP (Clotault *et al.*, 2010). It belongs to the family Poaceae with 2n=2x=14 chromosomes and has a genome size of 1.76 Giga bases (Varshney *et al.*, 2017). It is the staple food of Africa and North-west India, feeding about 90 million poor people across the world. India is the largest producer of pearl millet in the world that was grown in 6.93 million ha owing to average production of 8.61 million tonnes and productivity of 1243 kg/ha during 2018-2019 (AICRP, 2020. Pearl millet is considered to be a high energy cereal, rich in protein (8-19%), low starch content, high fiber content, rich in vitamins A and B, high calcium, iron, zinc with minor amounts of nutrients such as potassium, phosphorus, magnesium, copper, and manganese (Pattanashetti *et al.*,2016). In addition, it can be used as animal feed, brewery, and as roofing

material. Pearl millet is a plant with climate-smart vegetative, reproductive, and morphological features that makes the crop the ideal choice for the future (Taylor, 2016).

The genetic diversity of pearl millet is so wide, that it can be exploited and efficiently utilized for the development of new and economically superior varieties and hybrids. The present-day pearl millet varieties/hybrids development focused on meeting the nutritional quality requirement like Fe and Zn along with higher yield. There are about 66,682 accessions of pearl millet wild and cultivated germplasm across 65 countries in about 97 gene banks. ICRISAT gene bank in Hyderabad, India has the largest collection of accessions (https://www.genesys-pgr.org/c/pearlmillet). SSR markers are the most preferred marker type for fingerprinting, since they are very effective in distinguishing the germplasm. SSR markers have more advantages over the other markers due to its simplicity and higher reproducibility. SSR markers produces polymorphic genetic informations where the hyper- variable nature of the SSR marker produces very high allelic variations even among the closely related species (Vieira *et al.*, 2016). Many SSR markers have been previously developed and utilized for varietal/hybrid identification and marker-assisted breeding in many crops such as rice, maize, and sorghum whose genome resources are available. These markers are used in the study of genetic distance, evolutionary studies, construction of linkage maps and marker assisted selection, and defining cultivar specific fingerprints (Vieira *et al.*, 2016).

Many pearl millet varieties and hybrids are released for commercial cultivation from the Department of Millets, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University for the past 50 years. This study focuses on the most popular varieties and hybrids available in Tamil Nadu that has to be fingerprinted for varietal identification and germplasm registration with NBPGR. The EST-based markers that focused on fingerprinting belongs to PSMP markers series (Qi *et al.*, 2001 & Qi *et al.*, 2004), ICMP markers (Senthilvel *et al.*, 2008) and IPES series of markers (Rajaram *et al.*, 2013).

MATERIALS AND METHODS

Plant Materials

The Pearl millet cultivars *viz*,, CO (Cu) 9, CO 10, Pearl Millet Hybrid CO 9 along with its parents A'(male sterile) line ICMA 93111A and R'(pollinator) line PT 6029-30 were raised in the Department of Millets, Centre for Plant Breeding & Genetics, TNAU, Coimbatore during *Kharif*, 2019 in the yield trials. The TNAU pearl millet varieties used in the study are popular in Tamil Nadu due to its promising yield potential. The Co (Cu) 9 variety have special characters such as short duration (80- 85 days), high yield, and resistance to downy mildew. The composite variety CO 10 is developed by mixing and random mating of five elite inbred lines PT6029, PT6033, PT6034, PT6039, and PT6047 that is having a higher yield along with downy mildew disease resistance. Pearl Millet Hybrid CO 9 is a high yielding, early maturing hybrid developed from a cross between Cytoplasmic Male Sterile (CMS) line ICMA93111 (A line) and a pollinator line PT 6029-30 (R line). For which seedlings are planted on the side of the ridge and half way from the bottom. Depth of planting is 3-5 cm with the spacing of 45 x 15cm. Randomized Block Design (RBD) is followed with three replications.

Observation of Morphological Characteristics

The five randomly selected plants were subjected to morphological characterization to study the phenotypic performance under the plot size of 10 cents. The DUS characters for the 11 quantitative traits including plant height (cm), number of productive tillers, leaf sheath length (cm), leaf blade length (cm), leaf blade width (cm), days to 50% flowering, number of nodes (No's), spike exertion, spike length (cm), spike girth (cm), and 1000 seed weight (g) and 7 qualitative traits including anther color, node pigmentation, spike shape, node pubescence , spike density, seed color, seed shape were recorded.

Genomic DNA Isolation and PCR Analysis

Total genomic DNA was extracted from three-week-old leaves using the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray & Thompson, 1980). The isolated DNA samples were quantified with the micro-volume Spectrophotometer. Based on the quantity the DNA present in samples, it was diluted to a working concentration of 25ng/μL for the Polymerase Chain Reaction (PCR) amplification. The thirty-six primers were randomly selected based on the markers reported (Qi *et al.*, 2001 & Qi *et al.*, 2004; Senthilvel *et al.*, 2008; Rajaram *et al.*, 2013).The PCR (Eppendorf, Hamburg, Germany) reactions were performed to a total volume of 12-μL reaction mixtures containing 2 μL of Genomic DNA as a template, 7 μL of 1X Master Mix diluted from smart Prime Mastermix-Red (2X), 1μL of the primer pairs (Forward and Reverse) and 2 μL of Milli-Q water. The PCR profile consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of amplification at 94°C for 30 seconds of subsequent denaturation, 50-60°C for 30 seconds for annealing of primers to the template, and 72°C for 30 seconds for an extension. A final extension step at 72°C for 7 min was followed by termination of the cycle. The PCR amplicons were ranin 3% agarose gel prepared using a 1x TBE buffer at 100 V for 3 h. The resolved amplified products were visualized using a gel documentation system (Bio-Rad, CA, USA).

RESULTS AND DISCUSSION

The Pearl millet germplasm is characterized using morphological traits (Nehra *et al.*, 2016) for varietal and hybrid identification (Sumathi *et al.*, 2012). Most of the recent molecular markers studies in pearl millet focusing on allele richness and genetic diversity among the wild and cultivated plants. The hybrid purity tests and phylogenetic relationship analysis with the utilization of molecular markers such as RAPD (Randomly Amplified Polymorphic DNA) markers (Govindaraj *et al.*, 2009) and SSR markers (Kapila *et al.*, 2008; Waghmode, 2016; Chandra-Shekara *et al.*, 2017) are getting important because

of registration norms by the National Bureau of Plant Genetic Resources (NBPGR) and Protection of Plant Varieties and Farmers Rights Authority (PPVFRA).

Morphological Descriptors

The information outlined in Table 1 can effectively be used to find out distinct features of pearl millet cultivars. Plant height ranged from 80cm to 220cm for the cultivars under study. Among the cultivar, CO (Cu) 9 variety recorded the highest plant height (186-220cm) followed by pearl millet hybrid CO 9 (160-180cm) that is on par with CO 10 composite variety. Pearl millet hybrid CO 9 recorded the highest value for more than four quantitative characters. For instance, Number of productive tillers (4-6), Leaf blade length (60-68cm), Leaf blade width (4.0-4.5cm), number of nodes (8-10), and 1000 seed weight (13-14g) which is on par and comparable with the CO 10 composites and higher than that of the variety CO (Cu) 9. The highest leaf-sheath range was recorded in CO 10 composites (13.5 to 14.5 cm) and that is comparable with the pearl millet hybrid CO 9 (12.5- 13.5cm). For the traits spike length and girth, the highest value was recorded in the variety CO (Cu) 9 (33-39cm and 3-4 cm) followed by CO 10 composite (28-34cm and 3.1-3.6cm) and pearl millet hybrid CO 9 (25-35cm and 3.1-3.6cm). Eight qualitative traits were recorded and it showed that anther color was purple in pearl millet hybrid CO 9 which is distinct from the CO 10 (Yellow). Node pubescence is absent in the case of pearl millet hybrid CO 9 and is occasionally present in the composite variety CO 10, whereas glabrous in CO (Cu) 9 variety. Red color node pigmentation was observed in pearl millet hybrid CO9 and its parents viz., ICMA 93111A (Male), and PT 6029-30 (Female), and the composite CO 10 reflects green color node pigmentation. Spike shape and density are candle and compact in case of pearl millet hybrid CO 9 which is spindle/occasionally cylindrical and compact/rarely semi-compact for the composite CO 10 and CO (Cu) 9 it is a candle to cylindrical and the density is compact. Seed color is grayish-yellow for pearl millet hybrid CO 9 and CO 10 whereas, grayish seed with yellow base is recoded in CO (Cu) 9. Pearl millet hybrid CO 9 had a globular seed shape and it is recorded elliptical for CO 10, ICMA 93111A (Male), and PT 6029-30 (Female) (Table 1). Results were in accordance with (Sumathi *et al.*, 2012; Singh *et al.*, 2016).The Spike of the composites CO 10 (Figure 1A) and Pearl millet hybrid CO 9 (Figure 1B) (Sumathi *et al.*, 2017; Subbulakshmi *et al.*, 2018) is shown in Figure 1.

Fingerprinting of Pearl Millet Cultivars using SSR Markers

Most of the SSR markers were developed from the genomic and EST regions of the pearl millet genome. The Pearl millet SSR markers are derived from the conserved regions of the genome and hence easily differentiating the varieties/hybrids. A total of 36 SSR markers covering various chromosomes were taken for the initial polymorphism survey. All the 36 SSR primers (Table 2) showed proper amplification for the varieties *viz*., CO (Cu) 9, CO 10, Pearl Millet Hybrid CO 9, and its parents A' line ICMA 93111A and R'line PT 6029-30. Among the SSR makers, only two markers ICMP3021 and PSMP2089 distinguished the variety CO 9 from other pearl millet varieties. The markers ICMP3021 (Figure 2A) and PSMP2089 (Figure 2B) have

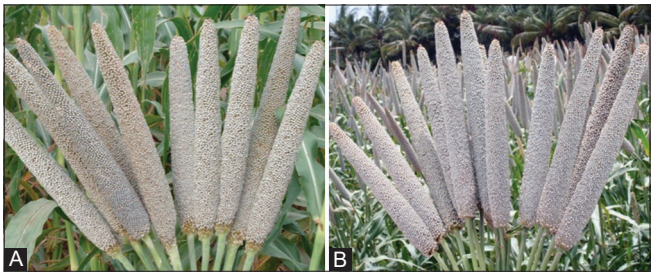

Figure 1: Spike of pearl millet hybrid and composites. 'A,–CO 10; 'B'– pearl millet hybrid CO9

Table 1: Morphological Characters of the cultivars screened

S. No	Characters	CO (Cu) 9	CO 10	TNAU Cumbu hybrid CO 9	ICMA 93111A (Male)	PT 6029-30 (Female)
1	Plant height (cm)	186-220	160-180	160-180	80-100	130-150
2	No. of productive Tillers	3-6	4-6	4-6	3-5	3-5
3	Leaf sheath length (cm)	--	13.5-14.5	12.5-13.5	10.5-12.0	12.0-13.0
4	Leaf blade length (cm)	--	55-65	60-68	45-50	65-75
5	Leaf blade width (cm)	--	4.0-4.5	4.0-4.5	2.5-3.0	3.8-4.3
6	Days to 50% flowering	50-55	47-50	45-50	45-50	50-55
7	Anther colour	--	Yellow	Purple	Yellow	Purple
8	No.of Nodes	--	7-8	8-10	4-5	6-8
9	Node Pubescence	Glabrous	Occasionally present	Absent	Absent	Present
10	Node Pigmentation	--	Green	Red	Red	Red
11	Spike exertion	Complete	Usually Complete	Complete	Complete	Partial
12	Spike Length (cm)	33-39	28-34	25-35	20-25	25-35
13	Spike Girth (cm)	3-4	3.1-3.6	3.1-3.6	2.1-2.5	3.4-3.6
14	Spike shape	Candle- Cylindrical	Spindle, Occasionally cylindrical	Candle	Candle	Candle
15	Spike density	Compact	Compact, rarely semi-compact	Compact	Compact	Semi-Compact
16	Seed colour	Grey seed with yellow base	Greyish Yellow	Greyish Yellow	Yellowish grey	Grey
17	Seed shape	--	Elliptical	Globular	Elliptical	Elliptical
18	1000 seed weight (g)	9-11	12-13	13-14	11-12	13-14

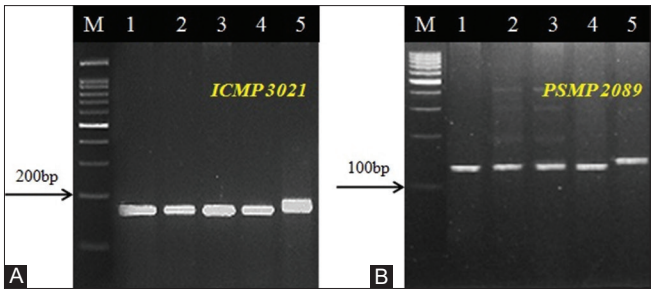

Figure 2: SSR marker analysis of pearl millet varieties and hybrid. 'M'–Ladder (100bp), '1'–PT 6029-30; '2'–ICMA 93111A; '3'–Pearl Millet hybrid CO 9; '4'– CO 10;' 5'–CO (Cu) 9

distinguished CO 9 variety by producing alleles at 200bp and 150bp, respectively. Whereas, other cultivars showed the alleles at 190bp and 130bp.

The composite variety CO 10 was discrimated from other lines using three markers *viz.*, ICMP3018 (Figure 3A), PSMP2219, and PSMP2220 by the presence of an additional allele indicating its composite nature. In the composite CO10, the marker ICMP3018 produces an additional allele at 210bp, the marker PSMP 2219 (Figure 3B) at 305bp, and the marker PSMP2220 (Figure 3C) at 128bp. None of the SSR makers helped for distinguishing TNAU Pearl millet Hybrid CO 9 from its parental

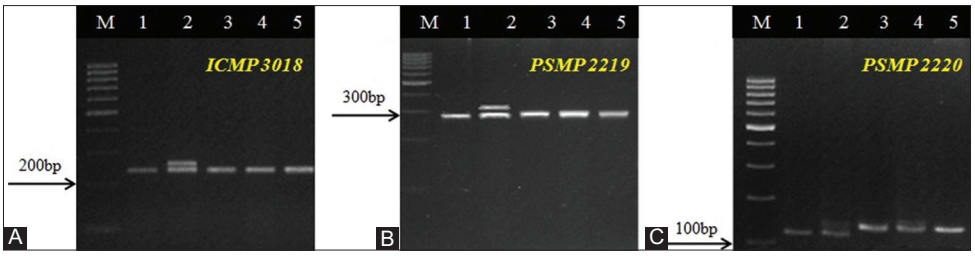

Figure 3: SSR marker analysis of pearl millet varieties and hybrid. 'M'–Ladder (100bp), '1'– CO (Cu) 9; '2'– CO 10; '3'–Pearl Millet hybrid CO 9; '4'– PT 6029-30;' 5'–ICMA 93111A

Table 2: Details of markers used for the polymorphic study

S. No	Marker name	Forward sequence	Reverse sequence	Annealing Temp.(°C)	Status of the allele	Allele size (bp)
1	ICMP3014	TGCTTCACAGCCTCTCCATA	CCACCATGCAACAGCAATAA	55	M	220
2	ICMP3018	ACGAGGACAAGCTCTTGGAA	ACGGCGCATACTCGATCATA	55	P	240
3	ICMP3021	GCCGACAGGAAGATTACGAT	AGCAAAACGCAGAACAACAG	55	P	200
4	ICMP3022	CTGGAAGTCCTTCTCGGTTG	CTGCTCCGCTCTGAATCTG	55	M	200
5	ICMP3033	GCCAAGGAGGTCAAGATCG	ACACGACTCGACTCAGACCA	58	M	200
6	ICMP3035	GCCAAGGAGGTCAAGATCG	ACACGACTCGACTCAGACCA	56	M	190
7	IPES0027	TGCTTGGGACAAAAGGCT	TAACTCAAGTGAGCGCAAGG	52	M	214
8	IPES0034	CCACAGGAGGAAAGAACACC	AGCACCGTGAACACAACAAC	50	M	176
9	IPES0037	GGGGGCTCACAGAACAAGTA	TCGGTTTGATTTTCTCCCAC	53	M	133
10	IPES0043	TGGATTGACGACTGGAATTG	GACTGACCAGGCACACCTTT	52	M	178
11	IPES0050	GCTCGGCAATCATGGAGTA	TTAATCCAGTGCTGCGTGTC	51	M	115
12	IPES0056	CGAAGAATGGATGGAATGAGA	CGAAGAATGGATGGAATGAGA	52	M	100
13	IPES0059	TTTCTTTGCGTTCTCCACCT	TTCTACGCATATAACCAGCCG	50	M	160
14	IPES0088	TGAGTGATCAACTGCAACTACC	CCATCACAACACTGGACAGG	51	M	191
15	IPES0094	ACTGTGGAGCAGAAGGGAAA	AACCAGTTCGGAAAGAGCCT	53	M	233
16	IPES0111	CTCGGAGGGTTCACCTTGTA	TGAGACCGTACTCCTGCTGA	51	M	264
17	PSMP2086	CGCTTGTTTTCCTTTCTTGCTGTT	CGCTTGTTTTCCTTTCTTGCTGTT	56	M	122
18	PSMP2087	GGAACAGACTCCATACCTGAAA	TACCTGCCTGTGCTGTTAGT	55	M	126
19	PSMP2089	TTCGCCGCTGCTACATACTT	TGTGCATGTTGCTGGTCATT	56	P	150
20	PSMP2202	CTGCCTGTTGAGAATAAATGAG	GTTCCGAATATAGAGCCCAAG	52	M	161
21	PSMP2204	TGCTTCTTGACTATGTTTTCC	AGATATGGCGAACGTGAGGAG	53	M	266
22	PSMP2205	AGGTGCTCACGAGCTGTAAGAG	AGCAAGACACTATTTTACCATC	55	M	202
23	PSMP2206	AGAAGAAGAGGGGGTAAGAAGGAG	AGCAACATCCGTAGAGGTAGAAG	57	M	203
24	PSMP2207	CAGGGCATACTTCAAGATTGATTC	GTCCACTTGTTATTCTCTATCACC	53	M	304
25	PSMP2209	TTGGACGATTTGGAAGCATAG	GAGGAAAAGAGCCATACAGAGAC	54	M	334
26	PSMP2210	CAATGATGACCGTAATCTGGGTG	GGGCAAGATATGTGAAATCAAG	53	M	313
27	PSMP2211	CTGCATGACGTGTGACCAATACC	AACAAATCAGCACCAGCCTCC	58	M	244
28	PSMP2213	CCCAAAAGAACCACACCCAC	GTTGATGCTACTGCTCGTTTG	55	M	197
29	PSMP2215	CCACGTCATTAGAGTAATCCGAG	ACTCAAATCCCAATCTTGAATC	52	M	238
30	PSMP2217	TTGTCTCTTCTCCGGCCAGATCCT	GCGTGATGTGTTTGGTACGCGAT	61	M	141
31	PSMP2219	ACTGATGGAATCTGCTGTGGAA	GCCCGAAGAAAAGAGAACATAGAA	56	P	305
32	PSMP2220	GCATCCTTCACCATTCAAGACA	TGGGAAACAGAATGGAGAAAAGAG	55	P	130
33	PSMP2221	TTGCCGTCAGCAATGTGCCT	CCGAAGTGCCCAGTGCCCAA	61	M	203
34	PSMP2222	TGGCTTCCAGACTAATCATCAC	TTATTTTAGCGGCGAGATTGAC	54	M	155
35	PSMP2223	CATGCTTCTTCTTCTTTTGTAACC	CAGCTCTTTGATCTCACTACAC	53	M	190
36	PSMP2225	CCGTACTGATGATACTGATGGTT	TGGGAGGTAAGCTCAGTAGTGT	56	M	239

(M) Monomorphic, (P) Polymorphic

lines. Hence, additional SSR makers have to be screened to distinguish the parental lines and hybrids.

CONCLUSION

In this study, morphological descriptors recorded using DUS characteristics and molecular screening for fingerprinting the pearl millet varieties, hybrids, and their respective parents were done. Nowadays, it is mandatory to fingerprint the recently released varieties/hybrids for the registration by NPBGR and also for the Protection of Plant Varieties and Farmers Rights Authority (PPVFRA). Thus, fingerprinting of varieties/hybrids using molecular markers and DUS characteristics paves a way for registration as well as for varietal identification.

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