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Molecular Analysis of Genetic Variability and Relationship among Pearl Millet (*Pennisetum glaucum*) Genotypes by Using SSR Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Pearl millet has a place with sort *Pennisetum*, and family *Poaceae*. Pearl millet is the most broadly developed grain crop in Asia and Africa representing close to half of the worldwide millet creation. In India, pearl millet is the fourth most broadly developed food crop after rice, wheat, and maize. We used molecular techniques to investigate the genetic diversity and relatedness of six genotypes *viz*:ICMV155, Dhanshakti, PC-612, Sampada, HHB-67, and HHB-197.the six genotypes was collected from National agriculture research project Aurangabad. The present study was conducted at the Department of Plant Biotechnology at K. K. Wagh College of Agricultural Biotechnology, Nashik.

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DNA was isolated by fixing a sample in alcohol without using liquid nitrogen; six genotypes were analyzed through SSR primers to determine the extent of molecular characterization. PCR amplification using 10 SSR primers generated a total of 111 no. of bands were scored corresponding an average of 11 bands per primer with 77 bands showing polymorphism (67%) and 34 bands showing monomorphism (30%). The PIC value ranged from 0.35 to 0.68 with an average of 0.4. Jaccard's coefficient based on SSR analysis 0.38 to 0.90. The dendrogram wasconstructed using the UPGMA method. It has two main clusters Cluster-1 consisting of C1- HHB 67, V6-ICMV-155, C2- HHB 197 and C4- Sampada. Cluster-2 comprised C3-Dhanshakti and V5-PC 612 as an out group. C2- HHB 197 and C4- Sampada genotypes have the highest similarity coefficient 0.76. Among all the genotypes, C1- HHB 67 and V5- PC 612 was found most diverse as it separated from all other genotypes at a very low similarity coefficient of 0.6. The identified markers can prove useful for identification of diverse germplasm and future DNA fingerprinting studies.

Keywords: Pearl millet; DNA; RNase; gel electrophoresis; SSR markers; DNA profiling.

1. INTRODUCTION

"Pearl millet [*Pennisetum glaucum* (L.) 2n = 2x = 14] commonly referred to as Bajra is otherwise called bulrush millet, cattail, or spiked millet" [1]. "Pearl millet belongs to genus *Pennisetum*, and family *Poaceae*. Pearl millet is the most broadly developed grain crop in Asia and Africa representing close to half of the worldwide millet creation. In India, pearl millet is the fourth most broadly developed food crop after rice, wheat, and maize" [2]. Pearl millet spikelets are uniquely protogynous, the majority of the styles have begun to evaporate before dust shed, so the yield is generally profoundly cross-pollinated. It is a double reason crop as it very well may be utilized as both feed and grain for domesticated animals. "The family *Pennisetum* contains around 140 species. The term 'millet' incorporates numerous types of different genera, among which the more conspicuous are pearl millet (*Pennisetum glaucum* (L.) R. Br.), Finger millet (*Eleusine coracana*. (L.) *Gaertn*), Foxtail millet (*Setariaitalica* (L.) Beauv) and Proso millet (*Panicum miliaceum* L.). Pearl millet assumes a basic part in food security" [3]. It has amazing properties like high photosynthetic proficiency, high dry matter creation limit, early development, dry season resilience, the necessity of insignificant buy inputs, and general liberation from biotic and abiotic stresses [2,4]. "It can endure the most unfavorable agro-climatic circumstances where different harvests like sorghum and maize neglect to create monetary yields" [2]. It can be designated as "Perfect resilient crop for the future" [5].In that capacity, it very well may be assigned as the "Ideal tough yield for what's to come" [5]. "The dietary benefit of pearl millet is considerably more prevalent than some other harvests since, it is profoundly nutritious, non-corrosive framing, no glutinous food having a few nutraceutical and useful well-being properties" [6,7]. "Pearl millet is the great wellspring of energy, carb, rough filaments (impervious to starch, solvent, and insoluble dietary strands), dissolvable and insoluble fat, proteins (8-19%), debris, dietary filaments (1.2g/100g), cancer prevention agents, fat (3-8%) with better fat edibility, iron, and zinc. It is likewise a rich wellspring of nutrients like riboflavin, niacin, thiamine, and minerals (2.3mg/100g) like potassium, phosphorous, magnesium, iron, zinc, copper, and manganese" [2]. "It displays a superior fundamental amino corrosive profile of protein in contrast with maize and rice. It contains lesser crosslinked prolamins prompting higher absorbability of the millet proteins" [8,9]. "It has 74% polyunsaturated unsaturated fat (PUFA) and wealthy in healthfully significant and crucial unsaturated fat like omega-3 unsaturated fats that is oleic corrosive (25%), linoleic corrosive (45%) and linolenic corrosive (4%) that are viewed as best for wellbeing. It is a gluten-free grain holding soluble properties even after being cooked and is hence really great for individuals having gluten energy. It contains a higher amount of gradually edible starch (SDS) and safe starch (RS) which represent a lower glycemic file (GI) and is quite pursued in the new seasons of changing weight control plans, food propensities, food industry, and diabetics. Pearl millet has a C: N proportion more noteworthy than 50. Pearl millet is tall and produces 3-5 tons/ac of dry matter biomass, like sorghum-Sudan grass. Rotting pearl millet establishes left on the dirt surface holding 60-80% of potassium in the straw for the accompanying harvest". (www.aicrp.icar.gov.in/pearl).

Pearl millet has higher fiber content. It goes about as probiotic nourishment for miniature

vegetation present in our body and along these lines gets us far from blockage [10,11]. It is additionally fit for bringing cholesterol down to cancer prevention agent movement with phytates, polyphenols, and so on (www.aicrp.icar.gov.in/pearl) These assume a critical part in counteraction of significant human illnesses like diabetes, malignant growth, and cardiovascular and neurodegenerative sicknesses [12]. Due to its various unique features, it is a very useful andi mportant crop for food and nutritional security and is rightly termed as nutri-cereal [13,14]. Because of its different extraordinary elements, it is an exceptionally helpful and significant harvest for food and nourishing security and is properly named nutri-oat [2]. In any case, yield is low and variable, seldom coming to 1000kg/ha. Besides, creation is compromised by fleece mold sickness, and striga parasitic weed predation by bugs [15]. India is the biggest maker of pearl millet on the planet with an area of 6.93 million ha., creation of 8.61 million tones, and an efficiency of 1243kg/ha (http://www.agricoop.nic.in). To bring millets into the standard for taking advantage of the nourishing rich properties and advancing their development, Govt. of India has declared year 2018 as the "Year of Millet" and the year 2023 as "International Year of Millets" by FAO committee on agriculture (COAG) forum. The significant pearl millet developing states are Rajasthan, Uttar Pradesh, Gujarat, Madhya Pradesh, Karnataka, Tamil Nadu, and Andhra Pradesh adding to 90% of complete creation in the country. The greater part of pearl millet in India is filled in the stormy season (Kharif) season (June/July-September/October). It is likewise developed during the summer season (February-May) in parts of Gujarat, Rajasthan, and Uttar Pradesh, and during the post-blustery (rabi) season (November-February) at a limited scale in Maharashtra and Gujarat.

"In the early days, crop breeders used morphological markers for the assessment of genetic diversity and choosing parents for developing new cultivars. Morphological characterizations were mainly used to select superior genotypes, but different studies revealed that morphological markers are not appropriate for traits exhibit in flower heritability. Morphological markers were greatly influenced by the interaction of the genotype with the environment in which it is expressed. Commonly known genotype environment interaction (GXE) occur when two or more genotypes perform differently in different environments,and are thus described as differential genotypic sensitivities to environments [15]. Moreover, due to the high out-crossing breeding nature and structure of genetic diversity in pearl millet species, the morphological data/markers are in adequate in providing, reliable information [12]. Such constraints gave rise to the evolution of molecular markers which cannot only be used to distinguish various germplasm accessions, but can also characterize and estimate genetic distances among different groups of genotypes ultimately enhancing the power of conventional plant breeding and genetics methods" [12]. "Thus, molecular markers put forward significant advantages over morphological markers for assessment of genetic diversity" [12].

"DNA based molecular markers have been successfully employed in quantification of genetic diversity, genotype identification, delineation and Marker assisted selection. These have been effectively utilized for the assessment of genetic diversity as compared to the morphological or biochemical due to their abundance, high level of polymorphism, reproducibility and being independent of the environment .As pearl millet exhibit tremendous amount of diversity at both phenotypic and genotypic level" [16]. Molecular markers namely RFLP,RAPD, ISSR and SSRs are mostly used, among the semicro satellite or Simple sequence repeats(SSRs)have been found as more reliable because they are highly polymorphic, informative, and co-dominant markers which are often broadly applicable since loci are frequently conserved between related species and sometimes. The integration of genomic technologies in pearl millet breeding William prove the efficiency of breeding programs in the development of better cultivars and reduce the time required for cultivar development. Thus, in the present investigation, SSR markers were used to characterize the pattern of diversity among Pearl millet.

2. MATERIALS AND METHODS

2.1 Plant Materials

Seeds of six genotypes (ICMV155, Dhanshakti, PC-612, Sampada, HHB-67, and HHB-197) of Pearl millet were procured from National agriculture research project, Aurangabad.

2.2 DNA Isolation and Purification

Total genomic DNA was carried from fresh leaf tissues by the method of Doyle and Doyle [17] with minor modifications as explained below.

The samples were prepared by putting approximately 200 mg of fresh leaf tissue into a mortar and pestle. The tissues were ground into fine powder. Thereafter, 600 µl of preheated plant extraction buffer was added and incubation of the tube was done at 65℃ for 60 min. After which the tubes were mixed by every 10 min of interval to homogenize the samples. The tubes were removed and allowed to cool, centrifuge at 10,000 rpm for 10 min at room temperature and the supernatant was then transferred into freshly labeled tubes. Then equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. Again, centrifuge at 10,000 rpm for 10 min at room temperature. Repeated the phenol: chloroform: isoamyl alcohol (25:24:1) treatment once. Aqueous layer was transferred into freshly labelled tubes and added equal volume of ice-cold isopropanol. Mix gently and incubate 4 ℃ for overnight to precipitate the DNA. It was then centrifuge at 10,000 rpm at 15 min at 4 ℃. The supernatant was decanted and added 500 µl of 70 % ethanol to wash the DNA palate, then centrifuged at 10,000 rpm for 15 min at 4 ℃. The supernatant was decanted and the palate was air dried until the ethanol smell disappeared, 20 µl of molecular graded water was added to resuspend the DNA. 2µl of RNasewas added and incubated at 37 ℃ for 45 min. In order to check the DNA quality and removal of RNA, 0.8 % gel was prepared by boiling 0.8 gm. of agarose in 100 ml of 1X TAE and allowed to cool at 55 ℃. Then 0.2 µl of etbr was added and gently mixed it. The solution was poured in the casting tray before it polymerizes.2 µl of DNA and 2 µl of loading dye were mixed together 4 µl of the mixture was loaded onto the 0.8 % agarose gel. The gel was run at 70 V for 45 min and the gel image was saved under the gel documentation system. When the protein and RNA was completely removed it was then proceeded to the Bio spectrometer.

2.3 SSR Amplification

A total of 20 primers were used in PCR amplification Table 1.SSR in this study were selected from the study of [5]. PCR amplification was carried out in 10 µl reaction volume containing 1.5 µl of template DNA (50ng), 1.6 µl *Taq* PCR buffer with $MgCl_2$(17.5 mM),0.3µl of *Taq polymerase* (1 U/µl), 1.5 µl dNTPs (2.5 mM) and 1 µl primer (10 pmol/µl).SSR PCR was performed at an initial denaturation 94 ℃ for 2.30 mins, 36 cycles of 94 ℃ for 30 sec, 55-60 ℃ for 1.30 min and 72 ℃ for 50 sec and final extension 72 ℃ for 7 mins.

Table 1. List of SSR primers

Sr. No	Primer Name	Sequence (forward and Reverse)	Annealing
1.	PSMP 2276	F-GGAATAGTATATTGGCAAAATGTG R-ATACTACACACTGTAAGCATTGTC	59℃
2.	PGIR D25	F- CGGAGCTCCTATCATTCCAA R- GCAAGCCACAAGCCTATCTC	55℃
3.	SSR-06	F-GCCTCGAGCATCATCATCAG R- CAACCTGCACTTGCCTGG	58℃
4.	PSMP 2070	F-ACAGAAAAAGAGAGGCACAGG R-GCCACTCGATGGAAATGTGAAA	55℃
5.	Xpsmp-2070	F-ACAGAAAAAGAGAGGCACAGGAG R-GCCACTCGATGGAAATGTGAAA	59℃
6.	Xpsmp-2084	F-AATCTAGTGATCTAGTGTGCTTCC R-GGTTAGTTTGTTTGAGGCAAATGA	58℃
7.	Xpsmp-2204	F- GAACTTGAYGAYGCCACTAGC R- TTGTGTAGGGAGCAACCTTGAT	59℃
8.	Xpsmp-2001	F-CATGAAGCCAATTAGGTCTC R-ACCATCTGACTTGTTCTTATCC	55℃
9.	PSMP-2018	F-CTCTGTAAGTTCCTGGTGCTCAA R-TCAGGCCAGTAACACATCTCAA	59℃
10.	UGEP-53	F-TGCCACAACTGTCAACAAAAG R- CCTCGATGGCCATTATCAAG	60℃

2.4 Agarose Gel Electrophoresis

Amplified products of SSR were analyzed on in 3.5 % Agarose in 1X TAE buffer and the bands were visualized on gel a Gel Doc system and documented.

2.5 Data Analysis and Scoring

Amplification profile of all cultivars compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band respectively. The data was analyzed using Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) software package. The dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

To compare the efficiency of primer, polymorphic information content (PIC) was calculated by using the formula

$$PIC = 1 - {}^{k}_{i=1}\sum P_i^2$$

Where Pi is frequency of allele for SSR marker.

3. RESULTS AND DISCUSSION

3.1 SSR Analysis

In the present study, six genotypes were studied with SSR markers. For SSR analysis among 20 primers tested, 10 primers generated 111 bands of these 77 bands were polymorphic with an average polymorphism of 67 %. Out of 10 primers, 4 primers (PGIRD 25, PSMP 2070, Xpsmp 2001 and UGEP 53) showed 100 % polymorphism similar results were reported by [2]. The molecular size of the amplified SSR products ranged from 200 bp (PSMP 2070) to 800 bp (PSMP 2076).The total no. of amplicons varied from 1 to 6 with an average of 3.5 per primer. The number of polymorphic amplicons ranged from 7 (PSMP 2018) to 19 (PSMP 2076) with an average of 13 bands per primerA similar study was reported by [3]. PSMP 2076 showed the highest PIC (0.69), while it was lowest for PSMP 2018 (0.37) with an average of 0.44 as mentioned in Table 2.

3.2 SSR-Based Cluster Analysis

Jaccard's similarity coefficients based on SSR markers among the all pair-wise combinations of genotypes ranged from 0.38 [Between Dhanshakti and HBB 197] to 0.90 [Between Sampada and HHB 67] with an average value of 0.72 as mentioned in Table 3.The dendrogram grouped into two main clusters on the basis of the reference line drawn at a similarity coefficient) In this dendrogram, two clusters were formed at the similarity coefficient of 0.62 which consisted of all the six genotypes of Pearl millet. Cluster-1 consisted of C1- HHB 67, V6-ICMV-155, C2- HHB 197 and C4- Sampada. Cluster-2 comprised C3-Dhanshakti and V5-PC 612 as an outgroup. C2- HHB 197 and C4-Sampada genotypes have the highest similarity coefficient 0.76. Among all the genotypes, C1-HHB 67 and V5- PC 612 was found most diverse as it separated from all other genotypes at a very low similarity coefficient of 0.6 as mentioned in Table 3.A similar study was reported by [3]. A similar type of report of his DNA fingerprinting of pearl millet using molecular markers has also been reported

Table 2. Sequence of SSR primer used for analysis of genetic divergence in pearl millet

Sr. No.	Primers	Total no. of bands	Total no. of Polymorphic bands	Total no. of Monomorphic bands	Polymorphism (%)	PIC value
1.	PSMP 2076	21	19	2	90%	0.69
2.	PGIRD 25	8	8	0	100%	0.36
3.	PSMP 2070	12	12	0	100%	0.62
4.	Xpsmp 2204	10	9	1	90%	0.53
5.	Xpsmp 2001	14	14	0	100%	0.63
6.	PSMP 2018	8	7	1	87.5 %	0.37
7.	UGEP 53	8	8	0	100%	0.47
8.	Xpsmp 2070	6	0	6	0	0
9.	SSR06	12	0	12	0	0.37
10.	Xpsmp 2084	12	0	12	0	0.37
Total		111	77	34	67%	0.44

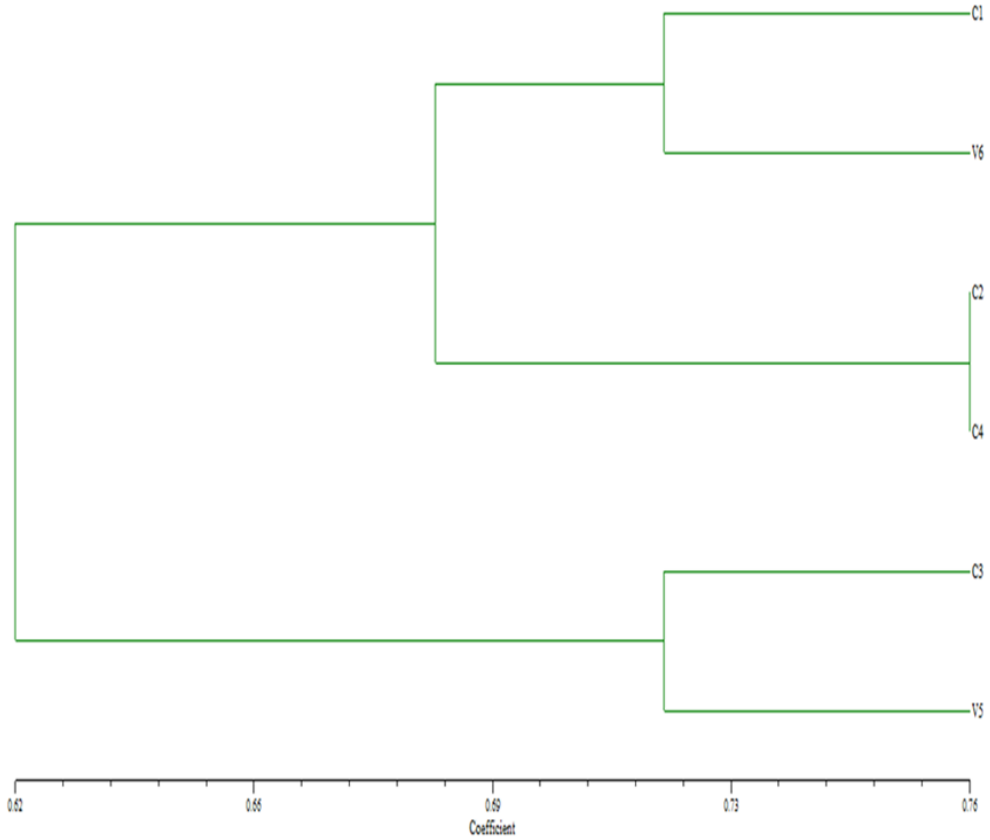

Fig. 1. Dendrogram of SSR marker

Table 3. Similarity index for the six pearl millet genotypes using SSR banding profile

Variety	HHB 67	HHB 197	Dhanshakti	Sampada	PC612	ICMV-155
HHB 67	1.0000					
HHB 197	0.62	1.00				
Dhanshakti	0.71	0.71	1.00			
Sampada	0.71	0.71	0.62	1.00		
PC612	0.71	0.71	0.71	0.90	1.00	
ICMV-155	0.66	0.38	0.48	0.48	0.48	1.00

by many other researchers [2] [3] and can be further used for cultivar identification purposes increase. The set of SSR markers used in this study could be used further and prove useful in positively assessing the ability of SSR to create unique DNA profiles in pearl millet. We used SSR for DNA profiling because microsatellites are considered suitable for cultivar identification due to their high accuracy and efficiency for detecting a large number of individual alleles [2] [18] Simple sequence repeats are tandem repeats of approximately 6 nucleotides in both coding and noncoding regions. SSR has become the marker of choice for genotyping because of its frequency, high allelic variation, codominant inheritance, and ease of analysis [12] [5]. Furthermore, microsatellite markers cannot be effectively applied to distinguish phylogenetically related species according to conserved sequences and may be useful in studying the genetic components of related species. There is [2] SSR fingerprints are generally highly discriminatory and are commonly used to identify breeds and individuals and to know their pedigree and identity. A DNA profile is required for submission of proposals, as entries promoted for third year consideration will be identified for clearance later. DNA fingerprinting data will be provided with the proposed plant variety registry. This will be of great help to a plant breeder when registering germplasm in his NBPGR. It can also be used to report the results of her DUS test with DNA markers.

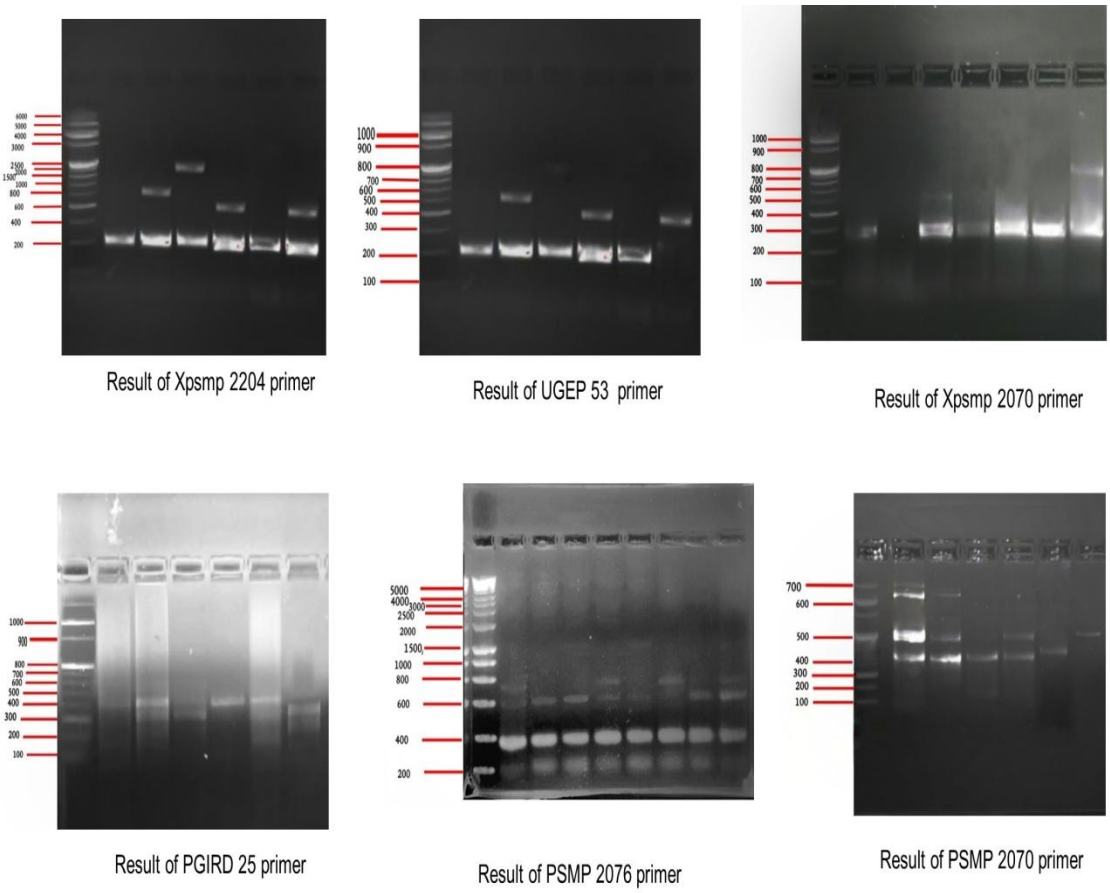

Plate 1. Gel photograph showing SSR allelic profile of pearl millet genotypes

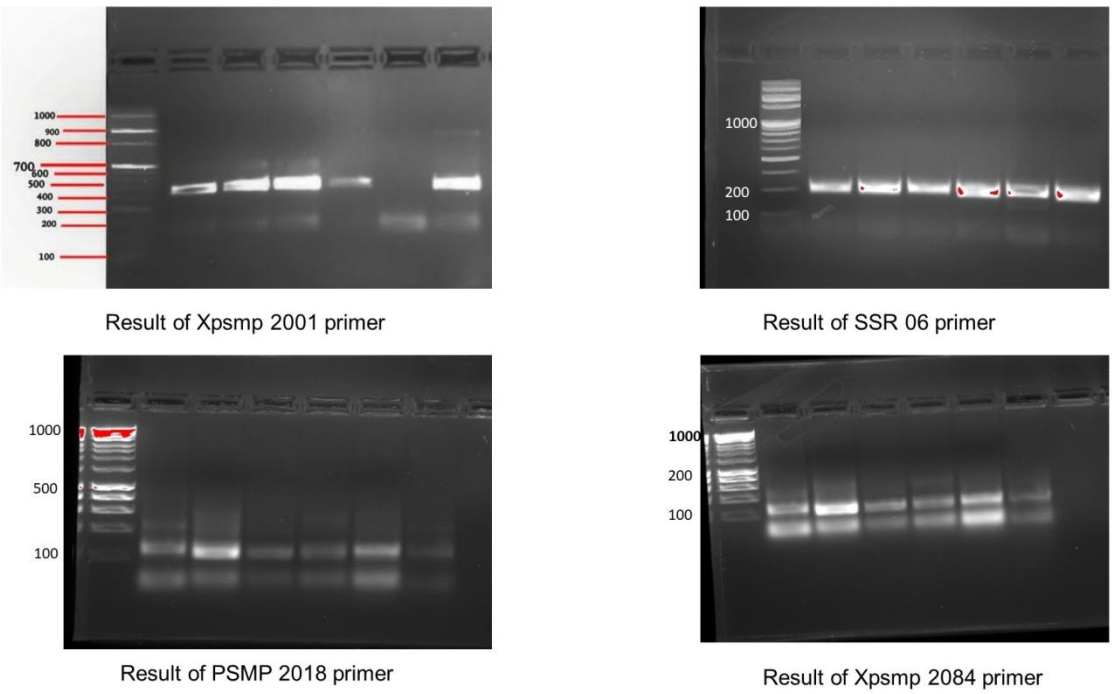

Plate 2. Gel photograph showing SSR allelic profile of pearl millet genotypes

4. CONCLUSION

The identification of primers that can apparently generate species specific profiles is significant for further phylogenetic studies in *Pennisetum glaucum*. SSR techniques indicated that it is useful in estimation of polymorphism and phylogenetic relationships among *Pennisetum glaucum* morphotypes. The data generated will be used for further breeding and characterization of the species. These findings not only highlight the capacity of the SSR technique but also helps in the selection of diverse Pearl millet landraces for conservation and crop improvement.

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CONFERENCE DISCLAIMER

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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