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Molecular characterization of Pearl millet (*Pennisetum glaucum* L.) genotypes

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Abstract
Pearl millet is the fourth most important food crop of the world next to rice, wheat and sorghum and is grown for dual purpose as grain and fodder. Downy mildew is one of the most widely spread and destructive disease of pearl millet, potentially resulting decline in production of pearl millet in India and western Africa. A major advantage of using molecular markers and biochemical marker for the screening the genotypes against disease at laboratory level will save time to screen the genotype compared to that of under field condition.

The main target of the ongoing research was to screen all genotypes to identify putative marker linked with the resistant and susceptible trait. In present investigation, eight downy mildew resistant genotypes *viz*., R15046, R15123, R15185, R15212, R15349, R15554, R15705, R16463 and three susceptible genotypes S21, S92, S7042 were screened by using SSR markers.

In SSR analysis data it was detected, the average 66.66% polymorphism with 0.197 PIC value per primer was detected. Xpsmp-2273 pearl millet specific primer amplified with ~200 bp size present in all eight resistant genotypes and found absent in susceptible genotypes showing 100% polymorphism with 0.318 PIC value. The genetic similarity values were retrieved using Dice coefficient for SSR data ranged from 0.33-0.92 with an average of 0.62. This primer has clearly differentiated the resistant and susceptible genotypes. This result further can be validated and utilized in QTL studies related with downy mildew resistance in pearl millet.

Keywords: Pearl millet, SSR, genotyping, cluster analysis

Introduction
Pearl millet is small seeded and drought tolerant crop found in tropical and sub- tropical regions of the world. The crop is grown very well in harsh environments where other crops usually fail (Abdalla *et al.*, 1998) [1]. Due to their ability to withstand drought, coupled with the production of substantial yields in a wide range of soils and climates, it is important high temperature and drought tolerant grain crop in Africa and Asia, particularly on low-fertility soils with limited water and nutrient holding capacity.

Recently, DNA simple sequence repeats (SSRs, known as microsatellites) are becoming the markers of choice to evaluate genetic characterization of genotypes against disease, fingerprinting and genetic diversity studies in a wide range of living organisms (Gupta and Varshney, 2000) [6] and offers an important DNA marker system because of their codominance, reproducibility, occurs in high frequency, locus specificity, distribution throughout the genomes of all higher plants and animals and multi-allelic characters. It displays a high level of polymorphism, even among closely related accessions, and is amenable to simple and inexpensive polymerase chain reaction (PCR) assays (Brown *et al*., 1996) [4]. The main advantage of SSR require a small quantity of DNA for PCR to detect polymorphism. Because of all these properties, SSR are suitable marker for screening a large number of breeding lines against disease (Senthivel *et al*., 2008).

The present study was conducted for molecular characterization of Pearl millet (*Pennisetum glaucum* L.) genotypes to assess the genetic diversity using SSR markers.

Materials and Methods
Plant materials
The experimental material included in present study consisted of pearl millet *(Pennisetum glaucum* L.) downy mildew susceptible and resistant pearl millet genotypes. (Table No. 3.1). The resistant and susceptible genotypes were screened and provided by National Agriculture Research Project, Aurangabad under Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani (MS).

Table 1: List of genotypes used in this study

Sr. No.	Downy mildew Resistant lines	Downy mildew Susceptible lines
1.	R-15046	S-21
2.	R-15123	S-92
3.	R-15185	S-7042
4.	R-15212	
5.	R-15349	
6.	R-15554	
7.	R-15705	
8.	R-16463	

DNA Extraction

High quality genomic DNA was isolated from young and fresh leaves (8-10 days old) of Pearl Millet (*Pennisetum glaucum* L.) by using Cetyl trimethyl ammonium bromide (CTAB) as per Doyle and Doyle (1987) [5] method with some modifications.

RAPD and SSR PCR Programming

DNA samples of all the samples were amplified using twelve SSR primers (Imperical Life Science) listed in Table No. 2. A 25 µl PCR reaction mixture contained 25 ng of genomic DNA, 1X PCR buffer, 0.25 mM dNTP, 1.7 mM $MgCl_2$, 1U *Taq* polymerase (Banglore Genei Pvt. Ltd.) and 10 pM each of forward and reverse primers. The DNA amplification was carried out in thermal cycler (Eppendorf® AG-22331). The optimized thermocyclic conditions for SSR amplification, an initial denaturation at 94ºC for 5 min followed by 30 PCR cycles (denaturing at 94ºC for 45 sec, primer annealing at 58-60 ºC for 45 sec and primer extension at 72 ºC for 1 min). A final extension step of 10 min and 8 min at 72 ºC was carried out for polishing the ends of PCR products.

Resolution of PCR product

Amplified PCR products were separated on 1.5% agarose gel (100V for 2.5 hr) using 100 bp DNA ladder (Banglore Genei Pvt. Ltd) as a molecular size standard. The gels stained with Ethidium bromide (10 mg/ml) were visualized and photographed under UV light in gel documentation system (Alphaimager™ 2200).

Data analysis

The Polymorphism Information Value (PIC) for SSR markers were calculated as, $PIC=\Sigma\ (1-P^2i)/n$, where n is the number of band positions analyzed in the set of accessions and P^2i is the frequency of i^{th} allele (Nei and Li, 1979). The data was used for similarity based analysis using the programme NTSYS-pc© (Version 2.02i) developed by Rohlf, 2000. The SIMQUAL programme was used to calculate the DICE coefficient and UPGMA cluster analysis.

Table 2: List of the SSR primers

Sr. No	Primers	Sequence 5'-3'	Tm (ºc)	GC (%)
1	Xicmp3088	F- TCAGGTGGAGATCGATGTTG	57.30	50.00
		R-TTACGGGAGGATGAGGATG	56.67	52.63
2	Xpsmp2255	F- CATCTAAACACAACCAATCTTGAAC	58.06	36.00
		R-TGCCACTCTTAAATTGACCAT	53.97	38.10
3.	Xpsmp2270	F- AACCAGAGAAGTACATGGCCCG	62.12	54.55
		R- CGACGAACAAATTAAGGCTCTC	58.39	45.45
4.	Xpsmp2227	F- ACACCAAACACCAACCATAAAG	56.53	40.91
		R- TCGTCAGCAATCACTAATGACC	58.39	45.45
5.	xpsmp2237	F- TGGCCTTGGCCTTTCCACGCTT	63.98	59.09
		R- CAATCAGTCCGTAGTCCACACCCCA	66.26	56.00
6.	Xpsmp2273	F- AACCCCACCAGTAAGTTGTGCTGC	64.43	54.17
		R- GATGACGACAAGACCTTCTCTCC	62.43	52.17
7.	Xpsmp2208	F- GAAAGAGCAAACTGAACAATCCC	58.87	43.48
		R- ACTTTGCCCTGGATGATCCTC	59.82	52.38
8.	Xpsmp2231	F- TTGCCTGAAGACGTGCAATCGTCC	64.43	54.17
		R- CTTAATGCGTCTAGAGAGTTAAGTTG	60.07	38.46
9.	Xpsmp2080	F- CAGAATCCCCACATCTGCAT	57.30	50.00
		R- TGCAACTGAGCGAAGATCAA	55.25	45.00
10.	Xpsmp2232	F- TGTTGTTGGGAGAGGGTATGAG	60.25	50.00
		R- CTCTCGCCATTCTTCAAGTTCA	58.39	45.45
11.	Xpsmp2031	F- CACATCCGCAAGAGACACCAAAT	60.25	47.83
		R- TTTGGGGGTGTAGGTTTTGTTG	58.39	45.45
12.	Xpsmp2089	F- TCCGGCGCTGCTACTTATAC	59.35	55.00
		R- TGTGCATGTTGCTGGTCATT	55.25	45.00

Results and Discussion

Total twelve pearl millet specific SSR primers were used. All primers generated satisfactory result to obtain 154 bands. Among 154 bands total number of polymorphic band was 110 and total number of monomorphic band were 44. The average polymorphism percentage of all SSR primers was 66.66% and the average of Polymorphim Information Content value was 0.197 per primer. The maximum number 16 bands were present in primer Xpsmp2232.

The percent polymorphism obtained for SSR primers were ranged from 0% (Xpsmp2237, Xpsmp2227, Xpmp2208 and Xpsmp2031) to 100% (Xicmp3088, Xpsmp2255,

Xpsmp2270, Xpsmp2273, Xpsmp2231, Xpsmp2080, Xpsmp 2232 and Xpsmp2089) with an average polymorphism percentage value of 66.66% per primer. The polymorphism Information Content (PIC) values for SSR marker were ranged from 0.000 (Xpsmp2237, Xpsmp2227, Xpmp2208 and Xpsmp2031) to 0.372 (Xpsmp2270) with an average PIC content value was 0.197 per primer presented in (Table no.3).

Table 3: Polymorphism pattern of SSR Primer

Sr. No.	Primer	Total no of amplicons	Total no of Polymorph IC amplicon	No of alleles	Allele size	Polymo RPHISM %	PIC Value
1.	Xicmp 3088	1	1	1	180	100	0.253
2.	Xpsmp 2255	1	1	1	200	100	0.151
3.	Xpsmp 2270	1	1	1	200	100	0.372
4.	Xpsmp 2237	1	0	1	220	0	0.000
5.	Xpsmp 2273	1	1	1	200	100	0.318
6.	Xpsmp 2231	1	1	1	150	100	0.151
7.	Xpsmp 2227	1	0	1	300	0	0.000
8.	Xpsmp 2080	1	1	1	300	100	0.318
9.	Xpsmp 2208	1	0	1	400	0	0.000
10.	Xpsmp 2232	2	2	2	300	100	0.263
11.	Xpsmp 2031	1	0	1	200	0	0.000
12.	Xpsmp 2089	2	2	2	300	100	0.355
	Average					**66.66**	**0.197**

The similarity matrix was used to generate dendrogram (Figure 4) through UPGMA cluster analysis by using NTSYS-pc (Version 2.02i). Based on dendrogram analysis of SSR fingerprint profile of eleven pearl millet genotypes were segregated into two main Clusters *viz*., Cluster-I and cluster-II (Fig. 1)

Cluster-I consisting of eight resistant (R15046, R15123, R15185, R15212, 15349, 15554, R15705, R16463) and with one susceptible (S21) genotypes and Cluster-II consisting of two susceptible genotypes (S92 and S7042). Cluster-I and Cluster-II shared 55% similarity.

Cluster-I divided into two subclusters *viz*., Cluster-A and Cluster-B. Cluster-A consisting of eight genotypes (R15046, R15705, R15123, R16463, R15185, R15349, R15212, S21) and Cluster-B separated alone as outgroup and consisting of only one genotype (R15554) and Cluster- A and Cluster-B shares 60% similarity with each other.

Cluster-A is further divided into two subclusters *viz*., Cluster-a and Cluster-b. Cluster-a consisting of (R15046, R15705, R15123, R16463) genotypes and Cluster-b consisting of (R15185, R15349, R15212, S21). Cluster-a and Cluster-b shares 86% similarity.

Cluster-a divided into two clades *viz*., Cluster-a1 and Cluster-a2. Cluster-a1 consisting of two genotypes (R15046 and R15705) and Cluster-a2 consisted of two genotypes (R15123 and R16463). Cluster-a1 and Cluster-a2 share 89% similarity with each other.

Cluster-b divided into two Cluster *viz*., Cluster-b1 and Cluster-b2. Cluster-b1 consisting of (R15185, R15349, R15212) and Cluster-b2 consisting of only one genotypes (S21). These two cluster shares 86% similarity with each other.

In present investigation the 12 SSR primers were used for screening downy mildew susceptible and resistant genotypes and exploited for the assessment of genetic diversity. The genotypes were categorized as downy mildew susceptible and resistant genotypes based on their trails conducted by NARP, Aurangabad. The aim of this study to screen these genotypes at primary level with SSR markers.

Thus, microsatellite markers used in the present investigation exhibited discriminating ability between downy mildew susceptible and resistant genotypes. Therefore, these primer were found to be useful for characterization of downy mildew susceptible and resistant and for the assessment of genetic diversity. The results were nearly in conformity with earlier reports of Bavadiya, 2014 [3] with different genotypes and primer.

Bavadiya, 2014 [3] revealed that total 80 primers gave satisfactory results among all SSR primers, Xicmp-3088, Xpsmp-2255, Xpsmp-2248, Xpsmp-2249, Xpsmp-2270, Xpsmp-2227, Xpsmp-2237, Xpsmp-2273, Xpsmp-2269 primer showed the amplification of unique and pearl millet gene specific bands in different genotype.

Nehra *et al*., 2017 [7] investigated that, the diversity among the 49 pearl millet inbreds and total 5 out of 29 polymorphic SSR loci, namely Xpsmp2070, Xpsmp2001, Xpsmp2008, Xpsmp2066, Xpsmp2072 revealed PIC values above 0.70, can be considered highly useful for differentiation of pearl millet inbred lines. The lowest PIC value (0.47) for linkage group 7 showed comparatively conserved nature of this linkage group A dendrogram obtained using WARD's minimum variance method further delineates 49 inbreds into 8 major clusters, and the clustering pattern collaborated with their pedigree and characteristics traits.

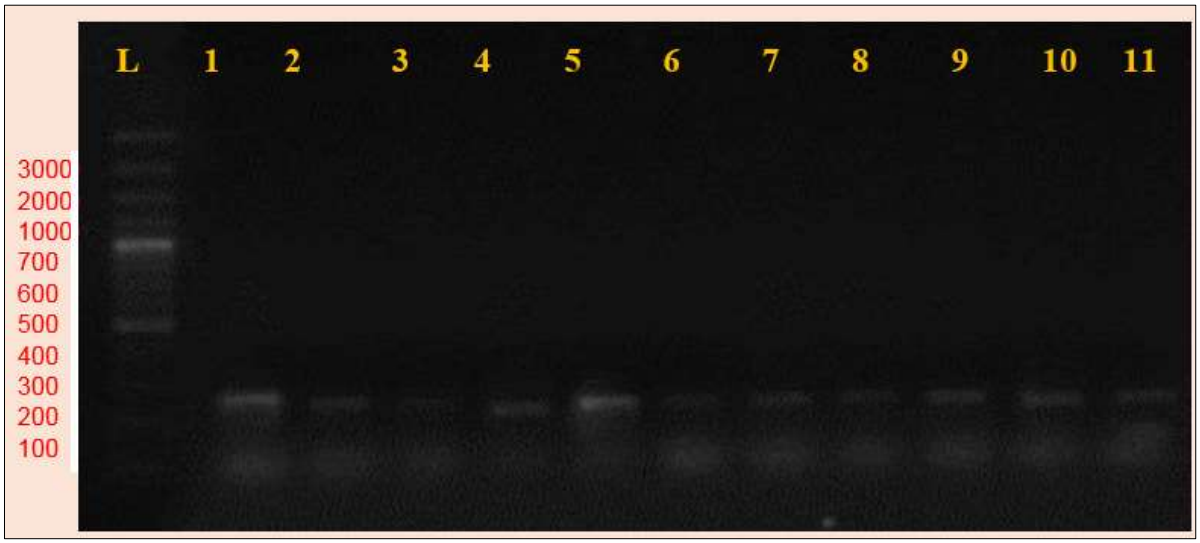


Fig 4.5: SSR profile of pearl millet genotype with primer Xpsmp-2237

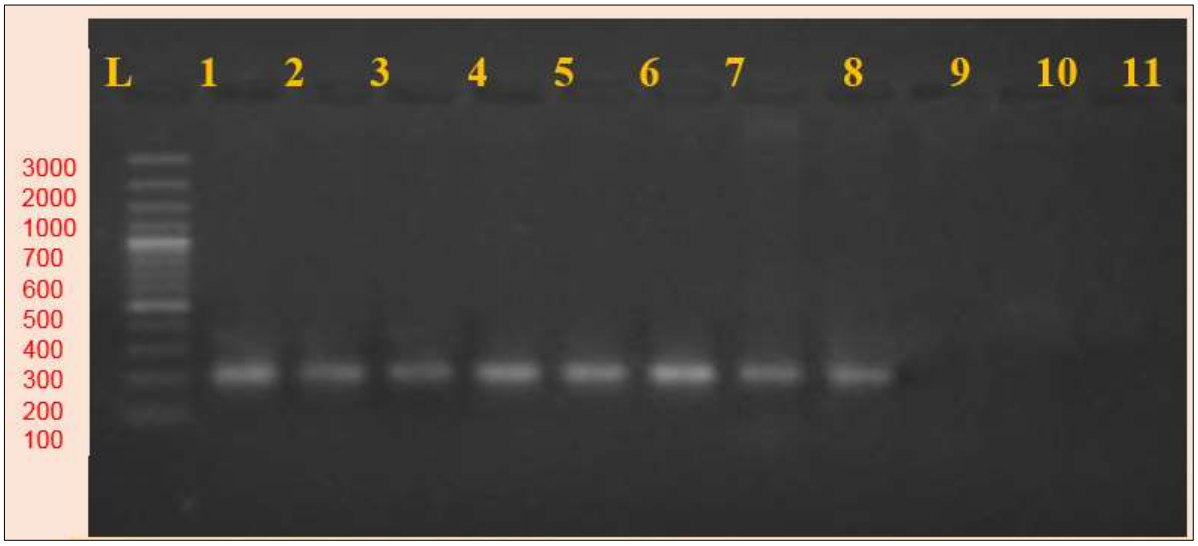
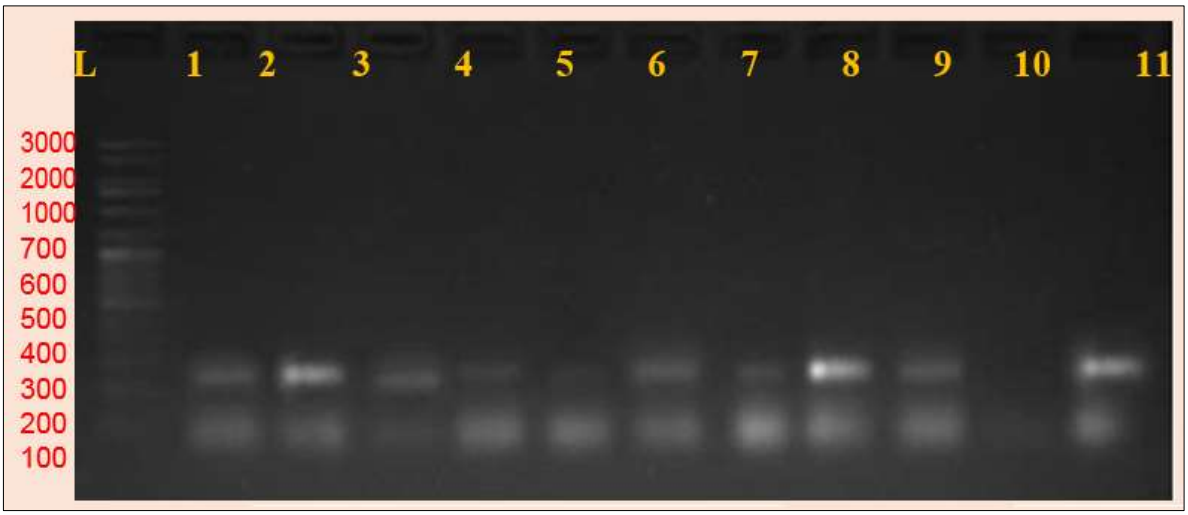


Fig 4.6: SSR profile of pearl millet genotype with primer Xpsmp-2273



1: R-15046
2: R-15123
3: R-15185
4: R-15212
5: R-15349
6: R-15554
7: R-15705
8: R-16463
9: S21
10: S92
11: S7042
L: Ladder

Fig 4.7: ISSR profile of pearl millet genotype with primer Xpsmp-2231

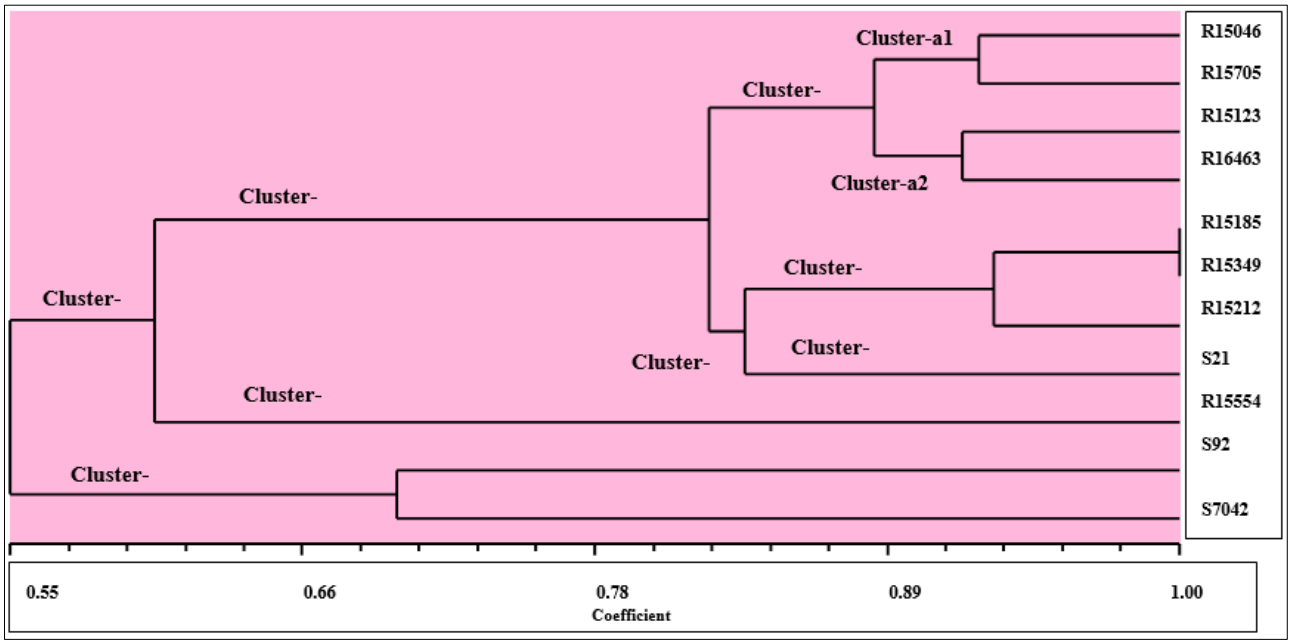


Fig 4.15: Dendrogram generated by UPGMA analysis based on SSR Primer

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