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Assessment of molecular diversity in pearl millet using SSR markers

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Abstract
Simple sequence repeats (SSR) analysis was used in pearl millet in order to assess the degree of polymorphism within and among genotypes and to investigate if this approach was suitable for genetic studies of pearl millet. The genotypes were evaluated using 28 SSR markers that were found to be polymorphic among the 50 SSR markers tested. The genetic distances were calculated using unweighted paired group method with arithmetic averages (UPGMA) method. A total 148 alleles were obtained with an average of 5.37 per locus. The polymorphic information content (PIC) values ranged from 0.74 (PSMP 2078) to 0.93 (PSMP 2214) with an average of 0.83. Comparison of the polymorphic products was generated by Jaccard's similarity co-efficient and using these values a dendrogram was generated which showed the phylogenetic relationships between the genotypes. The UPGMA analysis indicated a higher similarity between genotype ICMR 07999 and ICMB 98222 and lowest similarity index was observed between 670R and 2B. Analysis of SSR data helped in estimating genetic diversity and distinguish the genetic relationship among genotypes to identify the diverse genotypes which can be further used in hybridization programme.

Keywords: Simple sequence repeats (SSR), genetic diversity, pearl millet, polymorphic information content (PIC)

Introduction
Pearl millet is an allogamous cereal crop that belongs to the genus *Pennisetum* of family poaceae (gramineae) and has chromosome number 2n=14. It ranks 6th after crops like rice, wheat, maize, barley, sorghum and mostly grown in arid and semi-arid regions. In India it is cultivated in an area of 6.93 million ha with an average production of 8.61 million tons and 1,243 kg/ha productivity (Directorate of Millets Development, 2020) [5].

It is a climate resilient crop which can survive any adverse condition where no other cereal crop can be grown. It is generally referred to as a drought and heat tolerant crop. In India, it is mostly cultivated in Rajasthan, Gujarat, Uttar Pradesh, Maharashtra, Haryana and Tamil Nadu. It is also a nutri-cereal as it contains many vitamins and minerals and an assured source of nutrients in marginal areas contributing to food security.

The use of agro morphological parameters for the characterization of diversity is not sufficient and will not provide an accurate classification of the accessions, since morphological criteria are strongly influenced by the environment (Bahram *et al*., 2014) [3]. This is more pertinent to a cross-pollinated crop such as pearl millet. SSRs, also known as microsatellites, are repeated sequences of DNA and excellent tool to study the genetic relationship between closely related plant species. SSRs are being widely exploited in pearl millet because of their co-dominant inheritance, automated detection and high level of polymorphism etc. (Hernandez *et al*., 2002) [10]. SSRs are more abundant, ubiquitous in presence, hyper-variable in nature, easy to handle and have high polymorphic information content (PIC) making it extremely useful in crop improvement (Gupta *et al*., 2009) [8]. The objective of the present study was to evaluate genetic diversity among pearl millet genotypes using microsatellite (SSR) markers to identify genetically diverse genotypes of pearl millet which can be used in hybridization programme.

Materials and Methods
The experimental materials for study includes 150 genotypes (Table 1) obtained from ICRISAT, Patancheruvu and IIMR, Rajendranagar, Hyderabad. Molecular characterization was carried out at MAS laboratory, Indian Institute of Millets Research (ICAR-IIMR), Rajendranagar, Hyderabad during *Kharif* 2021.

Table 1: List of 150 pearl millet genotypes

S. No.	Genotype	S. No.	Genotype	S. No.	Genotype	S. No.	Genotype	S. No.	Genotype
1	2B	35	521B	69	584B	103	253R	137	ICMR 06555
2	4B	36	522B	70	586B	104	613R	138	ICMR 06777
3	6B	37	523B	71	588B	105	616R	139	ICMR 07444
4	8B	38	541B	72	591B	106	618R	140	ICMR 06222
5	10B	39	543B	73	593B	107	620R	141	ICMR 10555
6	12B	40	544B	74	594B	108	623R	142	ICMR 11888
7	14B	41	545B	75	595B	109	625R	143	ICMR 14888
8	22B	42	546B	76	596B	110	626R	144	ICMR 07999
9	26B	43	548B	77	ICMB 04999	111	629R	145	ICMR 08222
10	28B	44	549B	78	ICMB 92777	112	630R	146	ICMR 08888
11	32B	45	554B	79	ICMB 93333	113	632R	147	ICMR 13333
12	35B	46	555B	80	ICMB 94444	114	633R	148	ICMV 221
13	37B	47	556B	81	ICMB 94555	115	642R	149	RAJ171
14	38B	48	557B	82	ICMB 98222	116	644R	150	ICTP 8203
15	40B	49	558B	83	ICMB 99222	117	649R		
16	52B	50	559B	84	ICMB 00999	118	652R		
17	56B	51	561B	85	ICMB 97111	119	653R		
18	61B	52	562B	86	ICMB 95444	120	654R		
19	69B	53	564B	87	ICMB 142222	121	655R		
20	74B	54	565B	88	ICMB 17555	122	656R		
21	76B	55	566B	89	ICMB 91666	123	657R		
22	79B	56	567B	90	ICMB 01555	124	658R		
23	80B	57	568B	91	ICMB 09222	125	659R		
24	90B	58	569B	92	ICMB 13111	126	660R		
25	91B	59	570B	93	ICMB 13777	127	662R		
26	92B	60	571B	94	209R	128	663R		
27	97B	61	572B	95	214R	129	664R		
28	510B	62	573B	96	221R	130	669R		
29	511B	63	574B	97	228R	131	670R		
30	512B	64	575B	98	236R	132	674R		
31	515B	65	576B	99	243R	133	35R		
32	516B	66	577B	100	245R	134	R19		
33	517B	67	580B	101	247R	135	R37		
34	520B	68	581B	102	251R	136	R43		

DNA isolation: The genomic DNA was extracted from the young leaves of 15 days stage of pearl millet plant using CTAB (Cetyl Trimethyl Ammonium Bromide) method.

PCR Amplification: Among the 50 SSR markers used, some are PSMP series and two are ICMP series genomic SSR markers. All SSRs were screened against 150 genotypes. PCR was performed in 10 µl volume of sample containing 5 ng of DNA, 1pmol of each primer, 2 mMdNTP, 25 mM $MgCl_2$, 0.2 U Taq DNA polymerase and 0.5 µl of 10× PCR buffer (Applied Bio systems). PCR was performed by using PCR thermo cycler (Q-thermo cycler) using following conditions. Denaturation step of 3min at 94 °C followed by annealing step of 35 cycles with 1min denaturation at 94 °C, 1min of 54-62 °C and 1min of extension at 72 °C and final extension at 72 °C for 6min. Amplified fragments were separated on 3.0% polyacrylamide gels with Ethidium bromide using 0.5X TBE buffer at 220 V for 3 hrs. and band sizes were estimated with a 100 bp ladder and then recorded under UV in gel documentation system.

Data analysis

The gel pictures were scored for the presence and absence of amplified bands with 1 and 0 respectively, for all markers to generate a binary data matrix which was analyzed for genetic relationships among the genotypes using SIMQUAL module of software NTSYS-pc (version 2.02e) (Rohlf, 2000) [12]. Jaccard's similarity coefficients were calculated for all pairwise comparisons among all the genotypes. Based on the similarity values, UPGMA cluster analysis was performed to generate a dendrogram using SAHN module. The basic statistics such as polymorphic information content (PIC), allelic richness as determined by a total number of the detected alleles and the number of alleles per locus, gene diversity, and occurrence of unique alleles and heterozygosity (%) were estimated. The genetic diversity of each microsatellite locus was assessed by calculating frequency of the microsatellite alleles based on PIC using the formula, suggested by Anderson *et al.* (1993) [1]:

$$PIC = 1 - \sum_{f=1}^{n} (P_{ij})^2$$

Where, P_{ij} is the frequency of the j^{th} band for marker i, and summation extends over n^{th} band

Results and Discussion

A total of 50 simple sequence repeats (SSR) markers were used covering all the chromosomes of pearl millet for their molecular characterization and discrimination of 150 genotypes of pearl millet (Table 2). Out of 50 SSR, 28 markers were found to be polymorphic and a total of 148 alleles were detected in 150 genotypes of pearl millet. The number of alleles generated by each marker per locus ranged from 4 to 7 with average of 5.37 alleles per locus. The highest number of alleles (7) was observed in marker PSMP 2069 and PSMP 2233 and lowest number of alleles (4) was observed in PSMP 2248, PSMP 2078, PSMP 2246 and PSMP 2203. Similar results were reported by Satyavathi *et al.* (2013) [7],

Adeoti *et al.* (2017) [11], Yashveer *et al.* (2018) [9] and Kumar *et al.* (2020) [15]. The polymorphic information content (PIC) values ranged from 0.74 (PSMP 2078) to 0.93 (PSMP 2214) with an average of 0.83 (Table 2). This value is comparable to 0.69 (Elsi and Hancer 2014) [6] in maize but higher than 0.34 (Saxena *et al*., 2010) [13] in pigeon pea and 0.459 (Arya *et al*., 2014) [2] found for sorghum hybrids and parents. The range of PIC value showed the significance of locus specific PCR-based microsatellite markers and confirmed that SSR markers are highly elucidative and would be useful in hybrid breeding.

Table 2: Chromosome number, number of alleles and PIC values of 28 Markers

S. No.	SSR Markers	F/R	Sequence	Chromosome number	Number of alleles	PIC
1.	PSMP 2027	F	AGCAATCCGATAACAAGGAC	7	5	0.90
		R	AGCTTTGGAAAAGGTGATCC			
2.	PSMP 2068	F	CAATAACCAAACAAGCAGGCAG	1	5	0.80
		R	CTTCACTCCCACCCTTTCTAATTC			
3.	PSMP 2069	F	ACAGAAAAAGAGAGGCACAGGAGA	3	5	0.82
		R	GCCACTCGATGGAAATGTGAAATC			
4.	PSMP 2076	F	GGAATAGTTATTGGCAAAATGTG	4	7	0.86
		R	ATACTACACACTGTAAGCATTG			
5.	PSMP 2078	F	CATGCCCATGACAGTATCTTAAT	5	5	0.80
		R	ACTGTTCGGTTCCAAAATACTT			
6.	PSMP 2081	F	CTGTGCTGTCATTGTTACCA	4	4	0.74
		R	TCAGATCACCTATTACTTTCCCT			
7.	PSMP 2086	F	CGCTTGTTTTCCTTTCTTCTTGTT	4	5	0.89
		R	CCTTCTCAGATCCTGTGCTTTCTT			
8.	PSMP 2069	F	TGCATGAAAGTAGAGGATGGTAAA	2	5	0.85
		R	TGCATGAAAGTAGAGGATGGTAAA			
9.	PSMP 2089	F	TTCGCCGCTGCTACATACTT	2	5	0.78
		R	TGTGCATGTTGCTGGTCATT			
10.	PSMP 2090	F	AGCAGCCCAGTTTTACCTCAGC	1	5	0.75
		R	AGCCCTAGCGCACAACACAAACTC			
11.	PSMP 2202	F	CTGCCTGTTGAGAATAAATGAG	5	6	0.83
		R	GTTCCGAATATAGAGCCCAAG			
12.	PSMP 2203	F	GAACTTGATGAGTGCCACTAGC	7	6	0.83
		R	TTGTGTAGGGAGCAACCTTGAT			
13.	PSMP 2213	F	CCCAAAAGAACCACACCCAC	6	4	0.78
		R	GTTGATGCTACTGCTCGTTTG			
14.	PSMP 2214	F	CGCACAGTACGTGTGAGTGAAG	3	5	0.81
		R	GATTGAGCAGCAAAAACCAGC			
15.	PSMP 2224	F	GGCGAAATTGGAATTCAGATTG	7	4	0.84
		R	CGTAATCGTAGCGTCTCGTCTAA			
16.	PSMP 2225	F	CCGTACTGATGATACTGATGGTT	2	5	0.83
		R	TGGGAGGTAAGCTCAGTAGTGT			
17.	PSMP 2227	F	ACACCAAACACCAACCATAAAG	3	6	0.93
		R	TCGTCAGCAATCACTAATGACC			
18.	PSMP 2232	F	TGTTGTTGGGAGAGAGGGTATGAG	2	6	0.81
		R	CTCTCGCCATTCTTCAAGTTCA			
19.	PSMP 2233	F	TGTTTTCTCCTCTTAGGCTTCGTTC	5	5	0.88
		R	ACCTTCTCCGCCACTAAACAACT			
20.	PSMP 2237	F	CGGATGCTAAATTAACCGAAGC	4	6	0.82
		R	CCAGCTTGCTCTCGTTTCGTTC			
21.	PSMP 2246	F	TCTGTTTGTTTGGGTCAGGTCCTTC	1	7	0.83
		R	CGAATACGTATGGAGAACTGCGCATC			
22.	PSMP 2248	F	TCAAACATAGATATGCCGTGCCTCC	6	5	0.76
		R	CAGCAAGTCGTGAGGTTCGGATA			
23.	PSMP 2251	F	AAAGTGAATACGATACAGGAGCTGAG	3	4	0.75
		R	CATTTCAGCCGTTAAGTGAGACAA			
24.	PSMP 2263	F	AACCCCACCAGTAAGTTGTGCTGC	7	6	0.89
		R	GATGACGACAAGACCTTCTCTCC			
25.	PSMP 2273	F	CCAGTGCCTGCATTCTTGGC	1	6	0.88
		R	GCATCGAATACTTCATCTCA			
26.	PSMP 2275	F	TGGCCTTGGCCTTTCCACGCTT	6	6	0.83
		R	CAACCAGTCCGTAGTCCACACCCCA			
27.	ICMP 3002	F	AAGATGGATGATGGATTGATGA	6	5	0.90
		R	TACACACACATTGCCACACG			
28.	ICMP3027	F	ACACCATCACCGACAACAAA	5	5	0.90
		R	AGTGACCTGGGGTACAGACG			
Average					5.37	0.83

Jaccard's coefficient value ranged 0.72 to 0.94. Among 150 genotypes studied, the percent of similarity ranged from 63.51 to 93.90%. Genotypes near to low similarity values (63.51) are considered as most divergent genotypes and the genotypes close to high similarity values (93.90%) are considered to have more similarity between genotypes. The average similarity of all 150 genotypes is 63.51%. The genotypes ICMR 07999 and ICMB 98222 had maximum similarity 93.90% though they belonged to restorer and maintainer groups respectively while minimum similarity value of 63.51% was noticed between genotypes 670R and 2B. Similar studies were reported by Kumar *et al*. (2020) [15], Warrier *et al.* (2020) [14] and Chaudary *et al*. (2021) [4].

A dendrogram (fig.1) is formed using 28 SSR markers in 150 genotypes. The genotypes that were grouped together showed high similarity while the genotypes which are far away are considered to be divergent. Based on UPGMA and NTSYS-pc software of version 2.02, 150 genotypes are grouped (Table 3) into two major clusters I and II at 0.72 Jaccard's coefficient. Major cluster I again divided into two clusters IA and IB and again 1A is sub-grouped to two more clusters IA1 with 125 genotypes and IA2 with 7 genotypes. Advanced B lines, ICRISAT B and R lines and three OPVs are grouped in Cluster IA1 and it is the largest cluster with 125 genotypes.

Major cluster II is divided into two more clusters IIA with 9 genotypes and IIB with 5 genotypes. Cluster IB have 4 genotypes which are restorer lines. Cluster IIB have five restorer lines grouped. Restorer lines showed tall plants and early flowering compared to maintainer plants.

Table 3: Clustering pattern of 150 pearl millet genotypes based on molecular analysis

Main cluster	Sub Clusters	Sub-sub cluster	Number of genotypes	Genotypes
I	IA	IA1	125	94555B, 558B, 584B, 593B, 664R, 221R, 04999B, 566B, 569B, 571B, 575B, 581B, 588B, 591B, 594B, ICMR 17555, ICMR06222, ICMR 11888, 626R, 649R, 652R, 660R, 2B, 32B, 40B, 56B, 69B, 214R, 221R, 97111B, 245R, 247R, 251R, 253R, R43. 06777R, R19, 93333B, ICMB91666, 00999B, 09222B, ICMB 13777, 512B, 515B, 520B, 548B,574B,ICMR10555, ICMR11888, 663R, 08222R, 13111B, 564B, 568B, 623R, 4B, 8B, ICMV221, 38B, 90B, 91B, 236R, 247R 521B, 522B, 541B, 543B, 549B, 561B, 562B, 565B, 577B ICMR17555, 596B, 625R, 653R, 655R, 12B, 79B, 92B, 243R, 674R, 92777B, 94444B, 08888R, 06555R, 07999R, R37, ICMR14888, 13333R, 98222B, 01555B, 510B, 511B, 516B, 517B, 523B 544B 545B, 555B 556B 557B 566B 569B, 571B, 575B, 580B, 581B, 588B, 591B, 594B, 642R, 649R, 652R, 658R, 660R, 2B, 6B, 22B, 26B, 32B, 40B, 56B, 69B, 74B, 76B, 80B, 209R, 214R, 97111B, 245R, 253R, 266R, R43, 04999B, 567B, 570B, 572B, 576B, 595B, ICMR06222, 629R, 633R, 670R, 14B, 97B, 10B, 35B, 37B, 52B, 61B, 251R, 07444R, ICTP 8203, 35R, 228B.
		IA2	7	6B, 26B, 28B, 595B, 613R, 616R, 618R.
	IB		4	656R, 658R, 659R, 662R.
II	IIA		9	546B, 549B, 554B, 559B, 657R, 642R, 619R, 623R, 620R.
	IIB		5	654R, 644R, 630R, 633R, 629R.

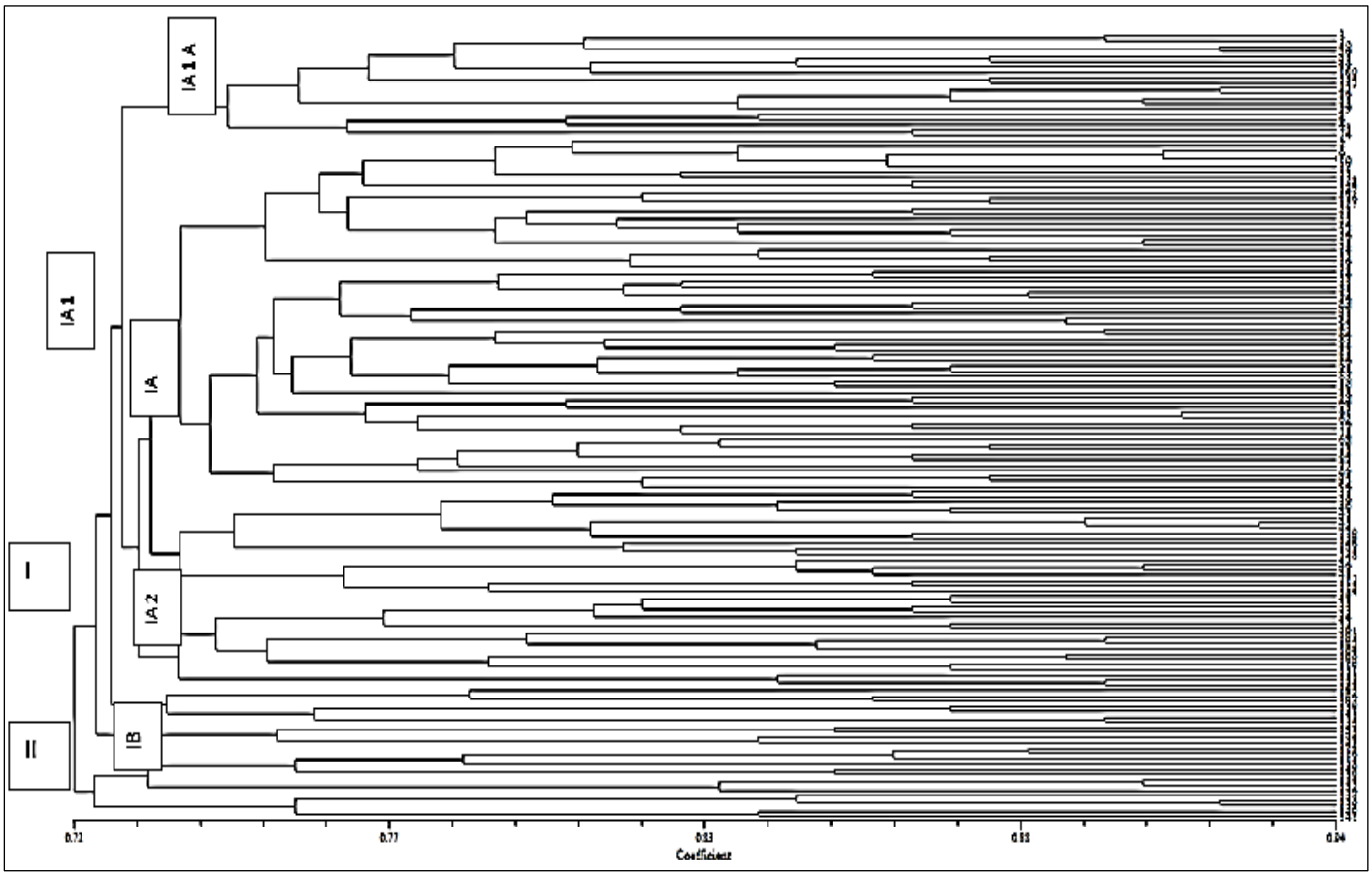
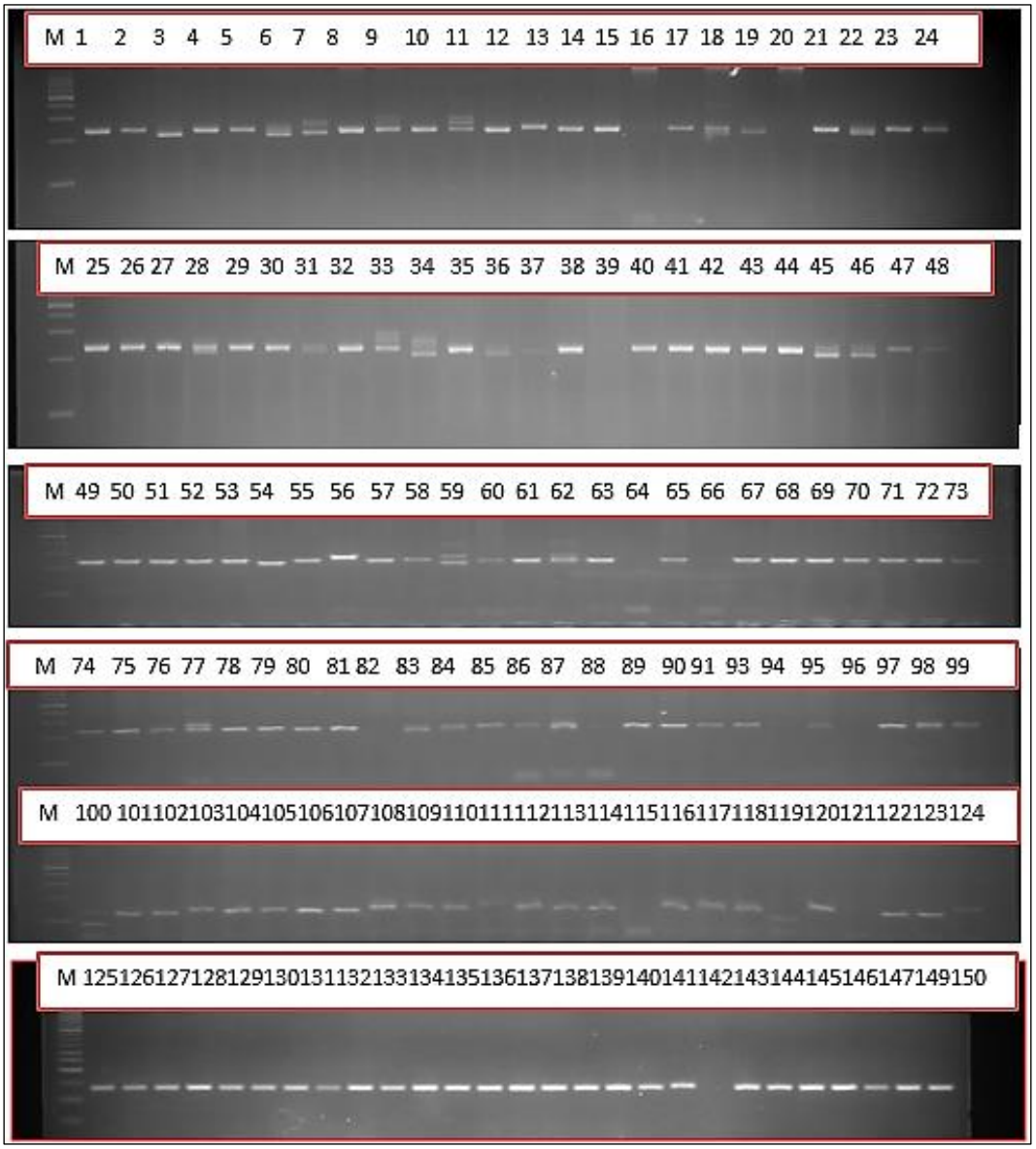


Fig 1: Dendrogram of 150 pearl millet genotypes



Gel image of marker ICMP 3027 for 150 genotypes.

Conclusion

SSR markers studied in pearl millet genotypes showed high genetic variability and these are considered as important molecular tool for estimating genetic diversity and similarities. The genetic relationships presented among the genotypes are helpful for future breeding programs (hybridization) through selection of genetically diverse parents. The results indicated from the present study can be helpful in selection, marker assisted selection (MAS) and crop improvement of pearl millet genotypes.

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