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Genetic diversity analysis of pearl millet genotypes for salinity tolerance through SSR marker at seedling stage

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Abstract
The experiment entitled on "Genetic diversity analysis of pearl millet genotypes for salinity tolerance through ssr marker at seedling stage" was conducted at Department Biotechnology, Junagadh Agricultural University, Junagadh during 2016-2017. The experimental material was comprised of 20 pearl millet genotypes and they were screened against different salinity levels for salt tolerance and susceptibility based on germination percentage. After screening, they were studied for polymorphism and the genetic diversity among salt tolerant and susceptible pearl millet genotypes using DNA based SSR molecular markers. Total 25 SSR primers generated total 72 fragments out of which 63 bands were polymorphic while no, monomorphic band were produced. The Polymorphism Information Content (PIC) values for SSR markers were ranged from 0.000 (Xpsmp-2236 and ICMP-3088) to 0.78 (ICMP-3081). The phylogenetic tree constructed by UPGMA method generated two main clusters and similarity coefficient was ranged from 11 to 69 %. SSR markers categorized salinity tolerant genotypes ICMA-98222, J-2480 and J-2340 and salinity sensitive genotypes ICMA-98444, ICMA-04999 and ICMA-96222 as most similar.

Keywords: SSR, PIC, tolerant, cluster

Introduction
Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a monocot with cross pollinating crop belonging to the family poaceae, having relatively small diploid genome (2n = 2x = 14) with DNA content of 1C of 2.36 pg (Budak *et al.,* 2003) [3]. Pearl millet is C4 species with high photosynthetic efficiency and locally known as bajra, is also known as bulrush millet, cat tail, spiked millet. Robust and free-tillering, pearl millet often grows to a height of three meters, although the most productive hybrids and improved open-pollinated varieties are often somewhat shorter than this. The plant stems are 10-20 mm thick and above each node is a shallow groove containing an axillary bud. These nodes are slightly swollen and they bear a ring of adventitious root primordia at the basal end. Leaves are flat, green and up to 8 cm wide. The grain-bearing head (ear) of the plant forms a compact, cylindrical, terminal, spike-like panicle. Pearl millet seeds are small, wedge-shaped to spherical and of various colours.
Currently, salinity is one of the most important limiting factor for crop production and becoming an increasingly severe problem in many regions of the world. Salinity is severe problem of germination in most crops. Screening of pearl millet varieties based on molecular markers and physiological parameters will be useful in improvement programmes for salinity tolerance in pearl millet varieties.
Pearl millet is a principal source of energy, protein, vitamins and minerals for millions of poor people in the regions where it is cultivated. It generally has 9 to 13 % protein but large variation among genotypes ranging from 6 to 21 % has been observed. It also contains approximately 71.6 % starch, 1.3% crude fibers, 5.1 % fat and 2.1 % soluble sugars. Pearl millet contains more calories than wheat, probably because of its higher oil content of 5 %, of which 50 % are polyunsaturated fatty acids. It is rich in calcium, potassium, magnesium, iron, zinc, manganese, riboflavin, thiamine, niacin, lysine and tryptophan. Pearl millet grain is gluten-free and thus, it is the only grain that retains its alkaline properties after being cooked which is ideal for people with gluten allergies. (Khairwal *et al.,* 2007) [6].

Material and Methods

The experimental materials for the present investigation consisted of 20 genotypes of Pearl millet [*Pennisetum glaucum* (L.) R. Br.]. These were obtained from the Pearl Millet Research Station, Junagadh Agricultural University, Jamnagar. The genotypes are listed in Table 1.

Table 1: Pearl Millet Genotypes use for screening against salinity tolerance

S. No	Genotypes	S. No	Genotypes
1.	J-2290	11	126-SM-15
2.	J-2340	12	74-SM-15
3.	J-2372	13	ICMA-04999
4.	J-2405	14	ICMA-05333
5.	J-2467	15	ICMA-94555
6.	J-2480	16	ICMA-96222
7.	J-2562	17	ICMA-97111
8.	J-2576	18	ICMA-98222
9.	J-2587	19	ICMA-98444
10	J-2588	20	ICMA-10444

Salt solutions were prepared by NaCl with different concentrations. The concentration of Saline Solutions 0, 50, 100, 150 and 200 mM NaCl were used as Salt stress treatments.

The experiment was carried out with Completely Randomized Design with Two Factors. The first factor was taken as genotypes [20 (twenty)] and second as salinity levels [5 (five)] with 3 replication.

Procedure of seeds germination

All 20 genotypes of Pearl millet [*Pennisetum glaucum* (L.) R. Br.] Were germinated. Seeds were sterilized with 0.1 % HgCl2 solution and washed with distilled water. Fifteen seeds of each genotype were kept in filter paper lined petri dishes and irrigated with water or salt solution in three replicates. These petri dishes were kept for germination under at room temperature in the laboratory condition. Care was taken that the filter paper remained moist and for that periodic additions of water or salt solution as per treatments were maintained.

- **Statistical analysis of germination percentage**

Statistical data analysis was carried out as per factorial completely randomized design. Analysis of variance was worked out using standard statistical procedures as described by Snedecor and Cochran (1967) [8].

- **Genomic DNA extraction**

DNA extraction protocol was standardized for ashwagandha from young leaves of 21 to 15 days old seedlings following CTAB (Cetyltrimethyl Ammonium Bromide) method of Doyle and Doyle (1990) [4] with some modifications by using PVP. The quality of DNA was checked on 0.8 % (w/v) agarose gel. The quantification of DNA was carried out using spectrophotometer. The good quality DNA samples with a ratio of 1.8 – 2.0 at O.D. 260 / 280 nm in spectrophotometric measurements were retained for PCR amplification.

- **SSR Analysis**

PCR amplification of the genomic DNA was carried out in 200 µl thin walled PCR tubes. The reaction volume of 15 µl was subjected to amplification through PCR in a thermal cycler (Eppendorf) along with the control (without template DNA). Each 15 µl reaction volume contained about 1.5 µl of 10X buffer, 1.2 µl of dNTPs, 1.2 µl of primer, 0.15 µl of Taq Polymerase, 9.75 µl of sterile distilled water, and 1.2 µl of total genomic DNA. The thermocycler was programmed as 4 min at 94ºC for initial denaturation, (1 min at 94ºC for denaturation, 2 min at 55ºC for annealing, 2 min at 72ºC for extension- 40 cycles), 10 min at 72ºC for final extension. PCR products were loaded on to 2 percent (w/v) agarose gel. The band profiles were visualized and documented using Alpha Innotech Flour Chem. FC2 gel documentation system.

- **Data scoring and analysis**

Data on SSR markers as presence or absence of bands of PCR amplified DNA fragments were scored. The data were subjected to statistical analysis for the calculation of Jaccard's similarity coefficient and cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using NTSYS-PC based software (Jaccard, 1908, Sokal and Michener, 1958 and Rohlf, 2000) [5, 9, 7]. The polymorphism percentage was calculated as per the method suggested by Blair *et al.*, (1999) [2]. Polymorphism information content (PIC) values were also calculated for each RAPD primer.

Result and Discussion

Effect of different salinity treatments on germination of pearl millet genotypes was tested and data are presented in Table 2 and Figure 1.

Increase salinity level observed drastic reduction in germination per cent and it was reduced by 58.5 % in T5 treatment as compared to control (T1).

Among the genotypes, mean germination per cent was significantly highest in genotype J-2290 (82.67 %), followed by genotypes ICMA-98222 (79.57 %) and J-2340 (76.89 %) while, it was significantly lowest with the genotype ICMA-05333 (48.44 %).

Among the salinity treatments, the mean germination percentage was recorded maximum with T1- 0 mM NaCl (89.99 %), while, significantly the minimum value was observed in the treatment T5 - 200 mM NaCl (37.34 %).

The same trend of results were also found in different groundnut varieties against increasing salinity in the experiment conducted by Ambede *et al*. (2012) [1].

Table 2: Effect of salt stress on germination of pearl millet genotypes.

S. No	Genotypes	Salinity Treatment					Mean
		T_1	T_2	T_3	T_4	T_5	
1	J-2290	95.53	86.70	84.47	77.77	68.90	82.67
2	J-2340	93.30	88.90	77.77	66.70	57.77	76.89
3	J-2372	97.77	93.30	77.77	62.23	42.23	74.66
4	J-2405	88.90	73.30	62.23	42.23	31.10	59.55
5	J-2467	93.30	84.47	64.47	60.00	55.53	71.55
6	J-2480	100	86.70	64.47	57.77	51.10	72.01
7	J-2562	95.53	73.30	77.77	55.53	42.23	68.87
8	J-2576	86.70	60.00	53.30	42.23	24.47	53.34

9	J-2587	100	66.69	57.77	33.30	22.23	56.00
10	J-2588	82.23	66.70	55.53	42.23	24.47	54.23
11	126-SM-15	93.30	75.53	71.10	48.90	35.53	64.87
12	74-SM-15	82.23	60.00	53.30	46.70	31.10	54.67
13	ICMA-04999	80.00	64.47	60.00	53.30	24.47	56.45
14	ICMA-05333	80.00	57.77	48.90	33.30	22.23	48.44
15	ICMA-94555	97.77	93.30	75.53	64.47	46.67	75.55
16	ICMA-96222	80.00	64.47	46.70	31.10	24.47	49.35
17	ICMA-97111	80.00	71.10	62.23	51.10	31.10	59.11
18	ICMA-98222	100	88.90	86.70	73.33	48.90	79.57
19	ICMA-98444	80.00	71.10	44.47	40.00	28.91	52.90
20	ICMA-10444	93.30	73.30	64.47	55.53	33.33	63.99
			S.Em. ±		C.D. at 5 %		CV %
G			0.87		2.43		5.27
T			0.43		1.22		
GXT			1.94		5.44		

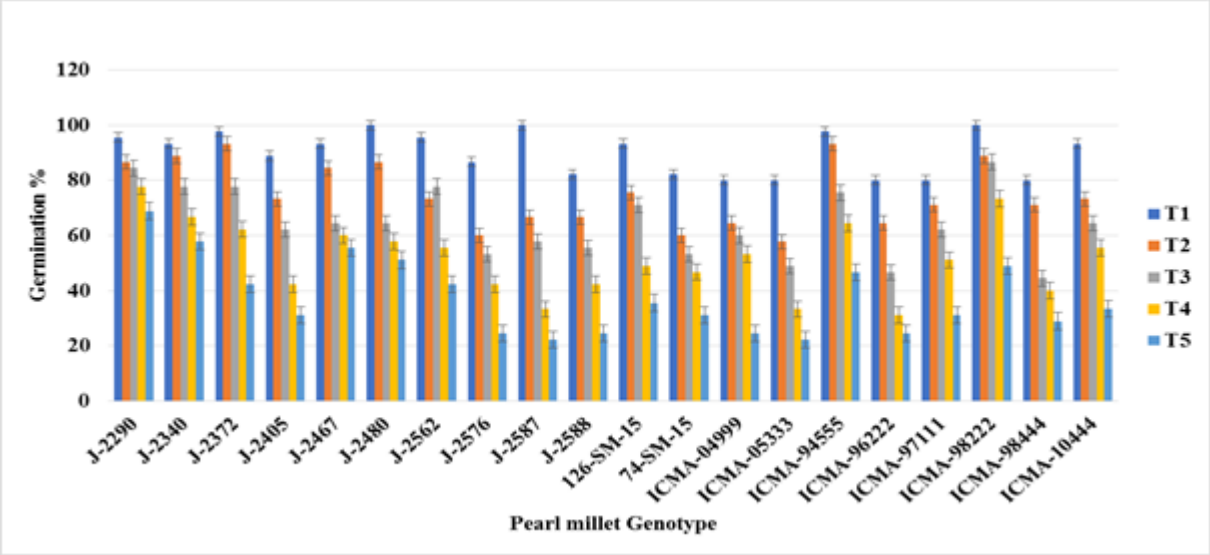

Fig 1: Effect of salt stress on germination of pearl millet genotypes.

Simple Sequence Repeat (SSR) Analysis

Total 25 SSR primers were used, all gave satisfactory results and generated 72 fragments. The SSR markers PSMP2267, ICMP3066, ICMP3081 and ICMP3093 produced highest number of 5 bands, while Xpsmp-2236 and ICMP-3088 produced lowest i.e. only one band. All the 72 bands were polymorphic with an average of 2.88 bands per primer and no band was monomorphic. Among the 72 polymorphic bands, 63 bands were shared polymorphic within two or more genotypes, while, 9 bands were unique-polymorphic (Table 4.15.).

The per cent polymorphism obtained for SSR primers were 100 % with an average value of 100 % per primer. The Polymorphism Information Content (PIC) values for SSR marker were ranged from 0.00 in ICMP3088 and xpsmp2236 to 0.78 in ICMP3081 primers with an average value of 0.48 per primer and SSR primer index (SPI) differed from 0.00 (ICMP3088 and xpsmp2236) to 3.88 (ICMP3081) with an average value of 1.59 as presented in Table 3.

Cluster Analysis of SSR

The dendrogram was constructed using UPGMA based on Jaccard's similarity coefficient through NTSYSpc v2.2 software for SSR data of 20 pearl millet genotypes (Table 4.17 and Figure. 4.13). The genotypes were grouped into two main clusters: cluster-I and cluster-II shared 11 % similarity. The cluster-I comprised of two subclusters A1 and cluster A2. Subcluster A1 consisted of one genotype J-2290 with similarity of more than 26 %. Subcluster A2 which further bifurcate into A2(a) and A2(b) with likeness of 31 % in which subcluster A2(a) consisted J-2340, J-2372, J-2576, J-2405, J-2588, ICMA-98222, J-2480, J-2562, J-2587, 126-SM-15, ICMA-98444, ICMA-10444, ICMA-04999 and ICMA-96222 genotypes while, subcluster A2(b) had only two genotypes with likeness of 47 % *viz.,* J-2467 and 74-SM-15. Cluster-II bifurcate into II(A) and II(B) with likeness of 23 % in which II(A) consistsed ICMA-97111 and ICMA-94555 genotypes while, subcluster II(B) consist only one genotype ICMA-05333.

Table 3: Size, number of amplified bands, per cent polymorphism and PIC obtained by SSR primers.

S. No.	SSR primers	Allele/Band Size (bp)	Total No. of Allele/bands (A)	Poly-morphic Bands			Mono-morphic Bands	% Poly-morphism	PIC*	SPI
				S	U	T				
1	PSMP2267	135-746	5	4	1	5	0	100	0.73	3.66
2	PSMP2078	177-237	3	3	0	3	0	100	0.57	1.70
3	PSMP2276	348-396	2	2	0	2	0	100	0.47	0.93
4	PSMP2018	212-281	2	2	0	2	0	100	0.17	0.34
5	ICMP3017	306-967	4	4	0	4	0	100	0.70	2.79

6	ICMP3024	172-207	2	1	1	2	0	100	0.12	0.25
7	ICMP3029	138-197	3	2	1	3	0	100	0.32	0.96
8	ICMP3043	212-244	2	2	0	2	0	100	0.49	0.98
9	ICMP3066	202-316	5	3	2	5	0	100	0.70	3.49
10	ICMP3078	301-976	3	3	0	3	0	100	0.63	1.88
11	ICMP3080	171-665	3	3	0	3	0	100	0.65	1.94
12	ICMP3081	262-329	5	5	0	5	0	100	0.78	3.88
13	ICMP3085	156-228	3	1	2	3	0	100	0.27	0.82
14	Xicmp3088	173-190	2	2	0	2	0	100	0.49	0.98
15	ICMP3092	221-316	3	3	0	3	0	100	0.64	1.93
16	ICMP3093	123-485	5	5	0	5	0	100	0.76	3.81
17	ICMP3096	250-271	2	2	0	2	0	100	0.48	0.97
18	ICMP4010	384-494	3	3	0	3	0	100	0.57	1.71
19	Xpsmp2225	188-275	2	2	0	2	0	100	0.50	1.00
20	Xpsmp2208	282-376	3	2	1	3	0	100	0.53	1.58
21	Xpsmp2248	188-208	2	2	0	2	0	100	0.47	0.94
22	Xpsmp2236	285	1	1	0	1	0	100	0.00	0.00
23	Xpsmp2219	289-329	2	2	0	2	0	100	0.42	0.83
24	M13 Xpsmp2237	174	4	3	1	4	0	100	0.63	2.52
25	ICMP3088	267-360	1	1	0	1	0	100	0.00	0.00
Total			72	63	9	72	0	-	-	-
Average			-	-	-	2.8	-	100	0.48	1.59

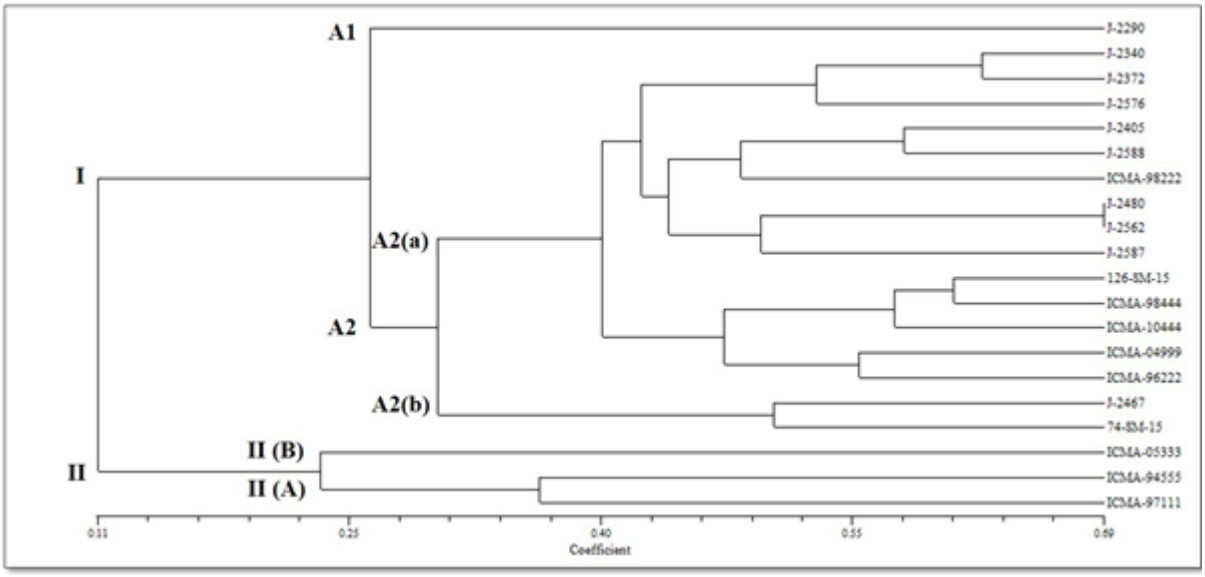

Fig 2: Dendrogram depicting the genetic relationship among 20 pearl millet genotypes based on SSR marker.

Conclusion

At high salt concentration (200 mM NaCl), germination percentage found maximum in J-2290 genotype followed by genotype J-2340. These genotypes showed their salt tolerance capacity according to their performance under salt stress. The minimum value for germination percentage was observed in ICMA-05333 and J-2587 genotypes.

SSR markers categorized salinity tolerant genotypes ICMA-98222, J-2480 and J-2340 and salinity sensitive genotypes ICMA-98444, ICMA-04999 and ICMA-96222 as most similar.

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