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Identification of Molecular Genetic Markers Associated with Salt Tolerance in Pearl Millet (*Pennisetum Glaucum* L.)

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ABSTRACT

Soil salinity is the principal abiotic stress that hinders plant growth and agricultural productivity. The salinity affects plant growth by the osmotic stress and ionic toxicity as well as ion imbalance. Therefore, it is necessary to screen germplasm with salt tolerance. In a randomized complete block design with four replications,five genotypes of pearl millet (*Pennisetum glaucum* L.) were evaluated under salinity soil durnig 2019 and 2020 seasons. The experiment was conducted at Sahl El-Hussinia, Agricultural Research Station, El-Sharkia Governorate, Egypt. This work aimed to evaluate five pearl millet genotypes under salinity stress based on yield, yield components, chemical and molecular analysis using the Inter-Simple Sequence Repeat (ISSR) marker. The results showed that the 2019 season had the highest values for all traits studied in this study compared to the 2020 season, and these values revealed high statistically significant differences between the genotypes of millet that were examined in the resistance to salt stress for different traits in both seasons. PE00463 genotype surpassed all other genotypes for plant height, number of tillers/m2 and total fresh dry forage yields, as well as crude fiber and crude protein Followed by PE00200 genotype, whereas the genotype PE000194 was recorded with the lowest values, indicating the presence of genetic variability within all genotypes to saline stress tolerance. A total of 56 ISSR bands were recorded, 27 and 29 of monomorphic and polymorphic bands, respectively. Three out of eight primers showed some molecular markers for salinity tolerance. Similarity relationships among pearl millet genotypes based on ISSR were ranged between 0.884 and 0.635. The dendrogram resulting separated the five pearl millet genotypes into two main groups and sub-main groups. The principal component analysis (PCA) has grouped the genotypes into three different groups that estimated 68.7% of the total variance in the values of the differences for the studied traits. The first and second principal components (PC1 and PC2) explained 40.4% and 28.3%, respectively. The heat map assignment analyses were used; the genotypes were distributed into two main clusters. Therefore, PE00463 and PE00200 genotypes can tend to new promising and more tolerant genotypes with salt stress.

Keywords: Chemical analysis, ISSR, quantitative trait loci (QTLs), pearl millet, and salinity stress.

1. Introduction

Pearl millet (*Pennisetum glaucum* L.) is a versatile cereal cultivated for food and forages; it has the capability to survive under the most adverse agro-climatic conditions compared to some other crops. The use of millet has a dual purpose as well as promising; the growth stages were early, and the crop is fast-growing up to several weeds. It has good salinity tolerance, and therefore such a plant is distinguished from other forage plants grown in salinity-affected areas. Pearl millet is a candidate for becoming a staple summer crop to ensure the quality of animal feed as well as human food in arid and semi-arid regions and elsewhere in the world under similar agricultural environments (Kulkarni *et al.,* 2006 and Patel *et al.,* 2008). Several researchers documented through their research papers that pearl millet showed minimal yield reduction in saline environments, indicating its salt-tolerant nature.

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(Makarana *et al.,* 2019). Besides, pearl millet has a marked ability to respond to favorable environments, thus making it an excellent crop for short growing seasons under improved crop management (Yadav and Rai, 2013).

From a nutritional point of view, pearl millet is nutritionally similar and even superior to major grains in terms of nutritional value in terms of chemical composition and therefore animal feed value. Millet is mainly included in the human diet as a grain crop in poor countries because of its high-quality chemical compositions, including dietary fiber and micronutrients (Sehgal and Kawatra, 2006). On the other hand, salinity stress is one of the most limiting factors in crop growth, which negatively affects agricultural productivity worldwide. It is worth noting that the salinity of agricultural soil means the high concentration of dissolved salts in the soil moisture in the root zone, through high osmotic pressure, which restricts the concentrations of dissolved salts on plant growth by reducing root water absorption (Ribadiya *et al.*, 2018). Moreover, salinity also affects plant growth because high salinity interferes with the plant's balanced absorption of essential nutritional ions. This explains the understanding of the means and path of salinity tolerance in crops, including the production of salinity-tolerant genotypes, which were vital plans to preserve the crop under salinity conditions of soil and water. In addition to producing salt-tolerant crops is a very necessary approach to overcoming the salinity threat (Rott and Shaw, 2001). To explain this, salt tolerance is one of the ways to increase the yield and productivity of crop plants in saline lands in their growth to become more tolerant of this salt stress. However, this goal of salinity tolerance in breeding programs faces many challenges, while the genetic payoff of many genes controlling the tolerance mechanism to stress simultaneous selection and selection procedures, especially under field conditions, presents another difficulty (Flowers, 2004).

Molecular markers have overcome the limitations of morphological markers in characterization by identifying variations directly from DNA. Molecular markers detected a high degree of polymorphism, evolutionary history, and germplasm conservation of pearl millet by several authors (Stich *et al.,* 2010; Adeoti *et al*., 2017; Gupta *et al.,* 2018; Hanaa & Nada 2018 and Jade *et al*., 2021). At the molecular genetic level, plants were tolerant of salt by activating regulatory and functional genes, thus molecular markers identify new sources and pathways for salt stress resistance in pearl millet. This is demonstrated by the recent ISSR molecular markers with excellent potential to aid genetic mapping through the selection of quantitative trait loci (QTLs) associated with economically productive traits. In addition, molecular marker-assisted breeding can provide an effective tool in identifying adapted germplasm. (Azzam et al., 2019; Abbas et al., 2021).Based on the foregoing, in this study, five pearl millet genotypes were evaluated for their ability to salinity stress tolerance and genetic variation using field evaluation and ISSR molecular marker analysis.

2. Materials and Methods

2.1. Plant material

Five pearl millet genotypes seeds were kindly obtained from International Crops Research Institute for Semi-Arid Tropics (ICRISAT), India and Forage Crops Research Department, Agricultural Research Center (A.R.C.), Egypt has presented in Table (1).

Table 1: Accessions names and sources of pearl millet used for the study.

No.	Accession	Collection Source	Accession Status
1	PE00056	ICRISAT	Natural
2	PE000194	ICRISAT	Natural
3	PE00200	ICRISAT	Natural
4	PE00463	ICRISAT	Natural
5	Shanduil-1	ARC (FCRD)	Natural

2.2. Field experiments

Two cultivation field experiments were conducted at Sahel Al-Hussainiya Saline Earth Station for Agricultural Research, Sharkia Governorate, Egypt, in the two successive seasons 2019 and 2020.

The analysis of soil chemical properties of the research station is illustrated in Table (2), according to Page *et al.* (1982).

Table 2: Soil chemical and physical properties of experimental site (Means of both seasons).

Particle size distribution (%)				Texture	O.M (%)	$CaCO_3$ (%)		
Coarse sand	Fine sand	Silt	Clay					
5.69	10.85	35.84	47.62	Clay	0.64	6.40		
pH (1:2.5)	EC(dSm^{-1})	Cations (meq/l)				Anions (meq/l)		
		Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO_3^{-}	Cl^{-}	SO_4^{--}
8.04	9.42	14.86	23.94	54.65	0.75	8.75	45.88	39.57
Macronutrients (mg/kg)			Micronutrients (mg/kg)					
N	P	K	Fe		Mn		Zn	
36.73	5.55	150	4.98		2.75		1.54	

The seed rate of the crop pearl millet was 20 Kg/fed. Sowing was done on 14, 20 May 2019, and 2020, respectively. All farming processes were carried out before planting. Superphosphate (15.5% P_2O_5) was applied at a rate of 100 kg fad^{-1} during tillage soil, ammonium nitrate (33.5% N) at the rate of 20 kg/fad was applied three times; 21, 45 and 65 days after planting. Potassium sulfate (48% K_2O) was applied at the rate of 75 kg K_2O fad^{-1} on two equal times 21 and 55 days after planting. Three cuts were taken through the growing season, the first cut was taken after forty-five days from sowing, and the other two cuts were taken consequently every thirty days.

2.3. The recorded data on each cut were as follows

- Growth traits:

- Plant height (cm).
- Number of tillers/ m^2.

- Fresh and dry forage yields:

- Fresh forage yield (kg/plot).
- Dry forage yield (kg/plot).

2.4. Chemical analysis:

Samples of each 10 gm cut oven-dried at 70°C for 48 hs up to the constant weight, ground, and prepwered for digestion as Page *et al.* (1982) description of an oven-dried powdered vegetable sample 0.5 g of each genotype digested with a mixture of H2SO4 and HCLO4 according to Chapman and Pratt (1961).

- The crude fiber (CF) contents were assessed according to Van-Soest *et al.,* (1991).
- The crude protein (CP): The nitrogen contents of the feed samples were determined by Kjeldahl N (A.O.A.C, 1999), and the value recorded for nitrogen was then multiplied by 6.25 (Hymowitz *et al.,* 1972).

2.5. Statistical Analysis:

All studied traits have been systematically analyzed for a randomized complete block design (RCBD) according to Gomez and Gomez (1984). Furthermore, the Bartlett test was performed to test for homogeneity. The least significant difference (LSD) was used at the 5% probability level.

2.6. Cluster analysis:

Using the arithmetic mean as described by Kovach (1995), a cluster analysis was performed, which was based on a similarity matrix (UPGMA). Moreover, the relationships of profiling were drawn on a genetic basis in the form of a tree diagram computed on the basis of the Joux-Cantor coefficient using the PAST program.

2.7. ISSR analysis:

From fiveteen primers successed eight primers for ISSR synthesized by (Operon Technologies, USA) were used in this study (Table 3). PCR amplifications were performed and countable according to Saha and Blumwald (2014) and using AlphaEaseFC version 4.0. (Alpha Innotech Corp., San Leandro, CA) softwwere.

Table 3: Names and their motifs of ISSR primers.

No.	Primer	Motif	No.	Primer	Motif
1	HB8	(GA)6GG	5	17898A	(CA)6AC
2	HB9	(GT)6GG	6	17898B	(CA)6GT
3	HB10	(GA)6CC	7	17899A	(CA)6AG
4	HB11	(GT)6CC	8	17899B	(CA)6GG

3. Results and Discussion

The data indicators presented in Tables 4 and 5 show that the first season was higher in the average values of all traits compared to second season, which announces the presence of statistically significant differences between the genotypes that were examined for salt stress tolerance for different traits in both seasons.

3.1. Growth traits

The effect of soil salinity on growth traits; plant height (cm) and number of branches/m^2 at different cuts of five pearl millet genotypes through both seasons (2019 and 2020) were presented in Table 4. Plant height and number of tillers were most important growth parameters relating to fodder green yield in pearl millet.

Table 4: Mean performance of five millet genotypes under soil salinity stress at different cuts over two seasons (2019 and 2020) on plant height and number of tillers traits.

Genotype	1st season (2019)							
	Plant height (cm)				Number of tillers /m^2			
	1st cut	2nd cut	3rd cut	Mean	1st cut	2nd cut	3rd cut	Mean
PE00056	98.32	103.25	93.99	98.52	69.42	72.73	65.85	69.33
PE000194	85.91	91.77	83.4	87.03	63.55	65.86	58.97	62.79
PE00200	109.47	112.74	104.84	109.02	72.31	74.62	67.74	71.56
PE00463	114.91	117.05	110.57	114.18	76.37	78.68	71.8	75.62
Shanduil-1	94.12	97.57	88.52	93.40	67.84	71.15	64.27	67.75
$LSD_{0.05}$	3.42	3.97	4.26	3.83	1.25	1.34	1.29	1.37
	2nd season (2020)							
	Plant height (cm)				Number of branches/m^2			
	1st cut	2nd cut	3rd cut	Mean	1st cut	2nd cut	3rd cut	Mean
PE00056	95.56	100.49	91.23	95.76	67.66	69.81	62.09	66.52
PE000194	83.71	87.73	80.36	83.93	60.51	62.82	55.94	59.76
PE00200	104.28	108.55	101.65	104.83	69.12	71.43	65.55	68.70
PE00463	111.02	113.16	106.68	110.29	72.48	74.79	67.91	71.73
Shanduil-1	91.78	95.23	87.18	91.40	65.5	67.97	60.93	64.80
$LSD_{0.05}$	3.05	3.21	2.95	3.28	1.14	1.26	1.07	1.36

They represent the vegetative growth stage and index, indicating the development of plants. The studied pearl millet genotypes were highly significant mean values for plant height (cm) and number of tillers/m^2 at the three cuts in both successive seasons, confirming the presence of genetic variability within genotypes to saline stress tolerance. The maximum values for the plant height (114.18 and 110.29 cm) were detected for PE00463 genotype. In comparison, PE000194 genotype had the lowest values

(87.03 and 83.93 cm) in both seasons, respectively, that probailites due to the variation in the abalities of tolerance in pearl millet genotypes to the saline stress. These results agree with those reported by Makarana *et al.* (2017) and Yazdizadeh *et al.* (2020), who were found highly significant variation among pearl millet genotypes in respect to height and number of effective tillers /plant. The second cut was the highest value in growth traits compared with the another cuts in all genotypes at the two growing seasons.

Also, data revealed that the PE00463 genotype ranked the highest, followed by PE00200 genotype in the number of tillers/m^2 (75.62 and 71.73) and (71.56 and 68.70) in both seasons, respectively. On the other hand, PE000194 genotype recorded the lowest values (62.79 and 59.76) in both seasons, respectively. These results were in the same data with those obtained by Reddy *et al.* (2021).

Naoura *et al.,* (2020) found highly significant differences among the growth traits of pearl millet genotypes under their study on the crop. So, plant height and number of tillers/m^2 were growth attributes directly linked with a genotype's productive potential (Maleko *et al.,* 2019).

3.2. Fresh and dry forage yields

Results in Table 5 demonstrate highly significant among the pearl millet genotypes to saline stress tolerant for fresh and dry forage yields at the three cuts in successive seasons, where it was ranged from 30.64 to 46.79 ton/fed and 4.59 to 7.10 ton/fed of the first season for both of total fresh and dry yields, respectively. In comparison, the total fresh and dry yields were ranged from 25.90 to 40.37 ton/fed and 3.87 to 6.47 ton/fed, respectively, in the second season.

It is worth mentioning that PE00463 genotype gave the highest values compared with the others genotypes of total fresh and dry yields (46.79 and 7.10 ton/fed) in the first season (2019). At the same time, it was recorded (40.37 and 6.47 ton/ fed) in the second season. These results were consistent with the finding of Toderich *et al.* (2018) and Jha *et al.* (2021), who reported that pearl millet line performance in response to different soil salinity levels varied. On the other hand, PE000194 genotype gave the lowest total fresh and dry yields values. In the same connection, Hajlaoui *et al*. (2021) found phenotypic variation between pearl millet-studied genotypes at different salinity levels and reported changes in forage yield resulting from saline stress.

Also, the results indicate that in both seasons, the heaviest fresh and dry yield was achieved at the second cut by PE00463 genotype (16.65–2.44 ton/fed, respectively in 2019 season) and (14.51-2.23 ton/fed, respectively in 2020 season), while the lightest values were shown at the third cut by PE000194 genotype (8.54–1.38 ton/fed, respectively in 2019 season) and (6.96- 1.14 ton/fed, respectively in 2020 season).

Table 5: Mean performance of five millet genotypes under soil salinity stress at different cuts over two seasons (2019-2020) on fresh and dry forage yields traits.

Genotype	1st season (2019)							
	Fresh yield (ton/fed)				Dry yield (ton/fed)			
	1st cut	2nd cut	3rd cut	Total	1st cut	2nd cut	3rd cut	Total
PE00056	12.48	14.76	11.77	39.01	1.73	1.92	1.61	5.26
PE000194	10.11	11.99	8.54	30.64	1.45	1.76	1.38	4.59
PE00200	13.76	15.98	12.83	42.57	2.03	2.34	2.13	6.50
PE00463	15.82	16.65	14.32	46.79	2.37	2.44	2.29	7.10
Shanduil-1	11.64	13.06	10 .08	34.78	1.84	2.17	1.78	5.79
$LSD_{0.05}$	0.573	0.416	0.653	0.698	0.094	0.076	0.078	0.159
	2nd season (2020)							
	Fresh yield (ton/fed)				Dry yield (ton/fed)			
	1st cut	2nd cut	3rd cut	Total	1st cut	2nd cut	3rd cut	Total
PE00056	10.94	12.52	9.23	32.69	1.59	1.92	1.53	5.04
PE000194	8.53	10.41	6.96	25.9	1.21	1.52	1.14	3.87
PE00200	11.73	13.95	10.8	36.48	1.84	2.15	1.94	5.93
PE00463	13.68	14.51	12.18	40.37	2.16	2.23	2.08	6.47
Shanduil-1	9.77	12.09	8.58	30.44	1.47	1.66	1.35	4.48
$LSD_{0.05}$	0.612	0.358	0.365	0.965	0.069	0.087	0.056	0.176

Pearl millet genotypes were varied in their tolerance to salt stress. Considerable variation in salt tolerance appears among pearl millet genotypes (Ashraf and McNeilly, 2006). In addition, Makarana *et al.* (2019) reviews some genotypes of pearl millet that have demonstrated superiority and can be adapted as a higher yield option than green forage in saline soil.

3.3. Chemical analysis

Crude fiber (CF) and protein (CP) were the most important quality features of forage crops under salt conditions which were of great importance for the production of high quality pearl millet from green forage crop.The perusal of data on crud fiber % and crude protein% were presented in Table 6, has reflected highly significant differences between the pearl millet genotypes under saline stress tolerance in both seasons. The mean maximum crude fiber content was recorded (28.46 and 26.41%) with PE00463 genotype. In contrast, the PE000194 genotype had the lowest value (23.39 and 21.31%) in both seasons. These results agree with Xie *et al.,* (2021), who observed genotypic variation for salt tolerance, and some genotypes displayed tolerance at crude fiber in Ryegrass genotypes. As well, the result recorded (11.22 and 10.25%) for PE00463 genotype that ranked the first, followed by PE00200 genotype (10.58 and 9.55%), while PE000194 genotype recorded the lowest value (9.98 and 8.89%) to crude protein% in both seasons, respectively. These results were substantiated by Daba *et al.* (2003), who indicate vast diversity between five Sesbania genotypes in fiber content and crude protein values under saline field conditions.

Table 6: Mean performance of five millet genotypes under soil salinity stress at different cuts over two seasons (2019-2020) on crude fiber and crude protein traits.

Genotype	1st season (2019)							
	Crude fiber %				Crude protein %			
	1st cut	2nd cut	3rd cut	Mean	1st cut	2nd cut	3rd cut	Mean
PE00056	26.26	28.08	25.03	26.46	10.42	10.60	10.21	10.41
PE000194	24.51	26.44	23.39	24.78	9.93	10.19	9.81	9.98
PE00200	27.31	29.29	26.24	27.61	10.53	10.77	10.43	10.58
PE00463	28.29	30.11	26.99	28.46	11.07	11.86	10.72	11.22
Shanduil-1	25.46	27.28	24.23	25.66	10.16	10.35	9.99	10.17
$LSD_{0.05}$	0.673	0.416	0.453	0.568	0.124	0.156	0.108	0.359
	2nd season (2020)							
	Crude fiber %				Crude protein %			
	1st cut	2nd cut	3rd cut	Mean	1st cut	2nd cut	3rd cut	Mean
PE00056	24.18	26.00	22.95	24.38	9.35	9.53	9.04	9.31
PE000194	22.54	24.36	21.31	22.74	8.86	9.09	8.74	8.89
PE00200	25.39	27.21	24.16	25.59	9.56	9.73	9.36	9.55
PE00463	26.21	28.03	24.98	26.41	10.32	10.79	9.65	10.25
Shanduil-1	23.38	25.2	22.15	23.58	8.98	9.25	8.81	9.01
$LSD_{0.05}$	0.787	0.504	0.465	0.498	0.112	0.132	0.126	0.136

3.4. Cluster analysis

A cluster among five pearl millet genotypes is present in (Fig.1). The dendrogram was generated using growth traits, fresh yield, dry forage yield, and chemical analysis data divided the pearl millet genotypes into two main clusters, cluster 1 separated into two subclusters. The first subcluster included PE00056 and Shanduil-1 genotypes and the second subcluster included PE000194 genotype only, while the second cluster was included PE00200 and PE00463 genotypes. These data reflect that different genotypes were genetically divergent, and the results were related with the morphological observations (Jha *et al.,* 2021).

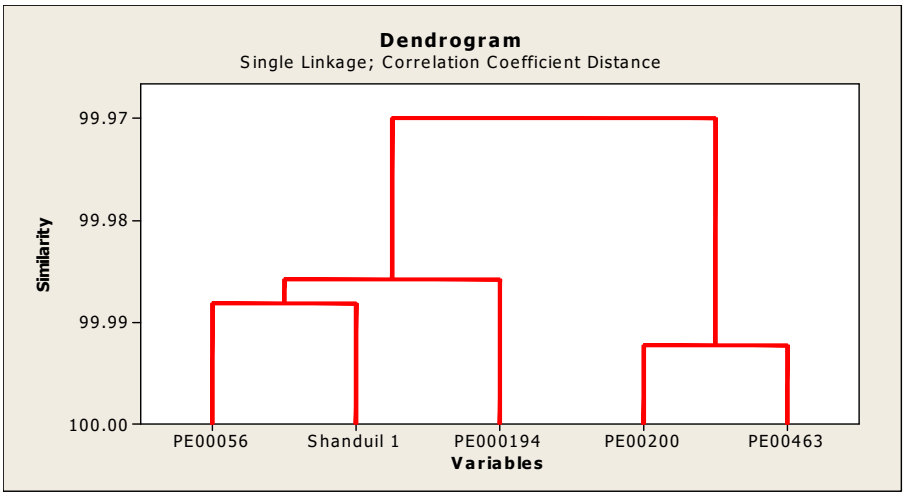

Fig. 1: Linkage dendrogram of five pearl millet genotypes generated by UPGMA cluster analysis based on growth traits, fresh yield, dry forage yield, and chemical analysis.

3.5. ISSR-primers molecular marker traits

ISSR technique was applied using eight primers to amplify the five genotypes of pearl millet. The analysis products involved 56 bands. There were 27 monomorphic bands, while 29 polymorphic bands (Table 7). The primer HB9 ((GT) 6GG) and HB10 ((GA) 6CC) produced the highest number of bands (9), whereas the primer17899A ((CA) 6AG) was showed the lowest number (5) with an average of 7 fragments per primer. The smallest number of polymorph loci or polymorphic bands (PB) was obtained with HB11 (1). The maximum number of PB was also recorded for HB8 (6 and 13, respectively). The incidence of polymorphism (%P) ranged from 16.66 (HB11 primer) to 75 (HB8 primer) with an average of 52.09%. This multiplicity was used to measure the tolerance difference for salinity between the genotypes studied. In this regard, Patil *et al.* (2018) mentioned that out of 288 scorable ISSR markers, 262 bands were polymorphic. On the other hand,the polymorphic bands were from 1 (UBC-825) to 7 (UBC 846), with an average of approximately 4 bands per primer. The heterozygosity (H) ranging from 0.01 (HB8) to 0.49 (17898B) with an average of 0.59 was observed. The Polymorphic Information Content index (PIC) values of ISSR data were between 0.01 and 0.37, with an average of 0.29. The lowest and highest PIC indices were recorded for HB11 and 17898B, respectively (Table 7).

Khaled *et al.* (2019) revealed average PIC values for ISSR markers were 0.43 in Sorghum Genotypes. In addition, Najaphy *et al.* (2011) reported that the moderate values of PIC for ISSR primers could be attributed to the diverse nature of genotypes and/or highly informative ISSR markers. The arithmetic mean of H (H.av) ranges from 0.01 (HB8, HB9, and HB10) to 0.03 (HB11) with an average of 0.01 (Table 3). The highest effective multiplex ratio (EMR, 7.50) was observed with the primer HB9, and the lowest amount of this ratio (3.25) was detected by the primer 17899A with an average EMR of 5.19 per primer. The marker index (MI) for ISSRs values ranged from 0.02 for HB11 to 0.07 for HB8 and 17898B, with an average of 0.05. The highest discriminating power (DP) value appewered with the primer 17898B (0.67), and 17899A observed the lowest DP as 0.31 with an average of 0.49 per primer. The highest resolving power (RP) value was for the primer 17899B (3.50), and primer 17899A observed the lowest RP as 0.50 with an average RP of 2.88 per primer. This study demonstrates the efficiency of ISSR markers as a genetic tool for selecting suitable accessions for breeding programs. Yadav *et al.* (2007) detected a total of 349 reproducible bands (73 monomorphic, 276 polymorphic) with a polymorphism rate of 79.1% in a genetic assessment study of pearl millet using 30 UBC#9 ISSR primer.

Table 7: ISSR primers, motifs, total band (TB), monomorphic band (MB), polymorphic band (PB), polymorphism percentage (P%), heterozygosity index (H), polymorphic information content index (PIC), effective multiplex ratio (EMR), Arithmetic mean of H (H.av), marker index (MI), discriminating power (DP) and resolving power (RP) as revealed by ISSR profiles in five pearl millet genotypes.

Primer	Motif	TB	MB	PB	p%	H	PIC_	EMR	H.av	MI	DP_	RP
HB8	**(GA)6GG**	8	2	6	75.00	0.47	0.36	5.00	0.01	0.07	0.62	3.00
HB9	**(GT)6GG**	9	6	3	33.33	0.28	0.24	7.50	0.01	0.06	0.31	2.00
HB10	**(GA)6CC**	9	4	5	55.55	0.42	0.33	6.25	0.01	0.03	0.52	3.25
HB11	**(GT)6CC**	6	5	1	16.66	0.01	0.01	6.00	0.03	0.02	0.41	1.00
17898A	**(CA)6AC**	7	4	3	42.85	0.29	0.25	5.75	0.01	0.06	0.33	2.50
17898B	**(CA)6GT**	6	2	4	66.66	0.49	0.37	3.50	0.02	0.07	0.67	2.00
17899A	**(CA)6AG**	5	2	3	60.00	0.46	0.35	3.25	0.02	0.04	0.59	0.50
17899B	**(CA)6GG**	6	2	4	66.66	0.41	0.33	4.25	0.02	0.06	0.51	3.50
Total		56	27	29	416.74	2.83	2.23	41.50	0.13	0.41	3.55	17.75
Mean		7	3.37	3.62	52.09	0.36	0.29	5.19	0.01	0.05	0.49	2.88

3.6. ISSR- analysis

The ISSR is based on molecular markers, on tandem repeats of short DNA sequences. These repeats secreted highly polymorphic variants in their molecular sizes resulting from chain interactions, and this occurs even between closely related or narrowly related genotypes due to the lack of functional and evolutionary constraints covering them at the DNA level. Furthermore, the DNA fragments covered in five pearl millet genotypes were considered under salt conditions using fifteen molecular names and eight ISSR primers succeeded (Fig. 2). number was developed by the HB10 primer (11 bands). Of the 59 sequencing reaction fragments that were amplified by the eight primers of the ISSR, 51 of them were polymorphic (86.4%). Moreover, Primer HB-8 showed 1618.41 base pair bands, which were found in PE00463 (salt-tolerant genotype), while they were absent in all other studied millet genotypes. Therefore, this range can be considered as a positive molecular marker for salt tolerance in pearl millet plants. In the other direction, there are no two bands with molecular size 1349.07 and 1094.57 base pairs and these two bands are considered to be the main point of the genotype imported PE00463 from Ecrysat organization. At the same time, it was investigated and presented in all other tested genotypes so that it could be used as a negative molecular marker for salinity tolerance in pearl millet. Then on the other hand, Primer HB-10 showed one positive and one negative molecular marker for salt tolerance in pearl millet with a molecular size of 642.07 and 327.9 bp, respectively. Primer 178988B was shown with a single strand with a molecular size of 758.83 bp, which was present exclusively in PE00463. Therefore, this strip can be used as a positive molecular marker for salt tolerance in pearl millet. Our results were in agreement with those of Younes *et al.* (2007), who used simple sequence repeat (ISSR) to distinguish environmental stress tolerance (salinity) in the field and obtained some genetic markers associated with salt tolerance in sorghum grain that could be used during the breeding program for this crop. and also the identification of genomic regions that determine yield under abiotic stresses, especially final salinity by Shivhwere and Lata, (2017), who used molecular marker-based genetic association maps and paved the way for molecular marker-assisted selection and selection and breeding of salt-tolerant genotypes in millet pearly; This shows that the above results show that the number of molecular markers related to salt tolerance reflects the complexities of this trait as well as the influence of environmental factors. However, marker-assisted selection or selection can enhance the identification of pearl millet genotypes that are tolerant of environmental stress.

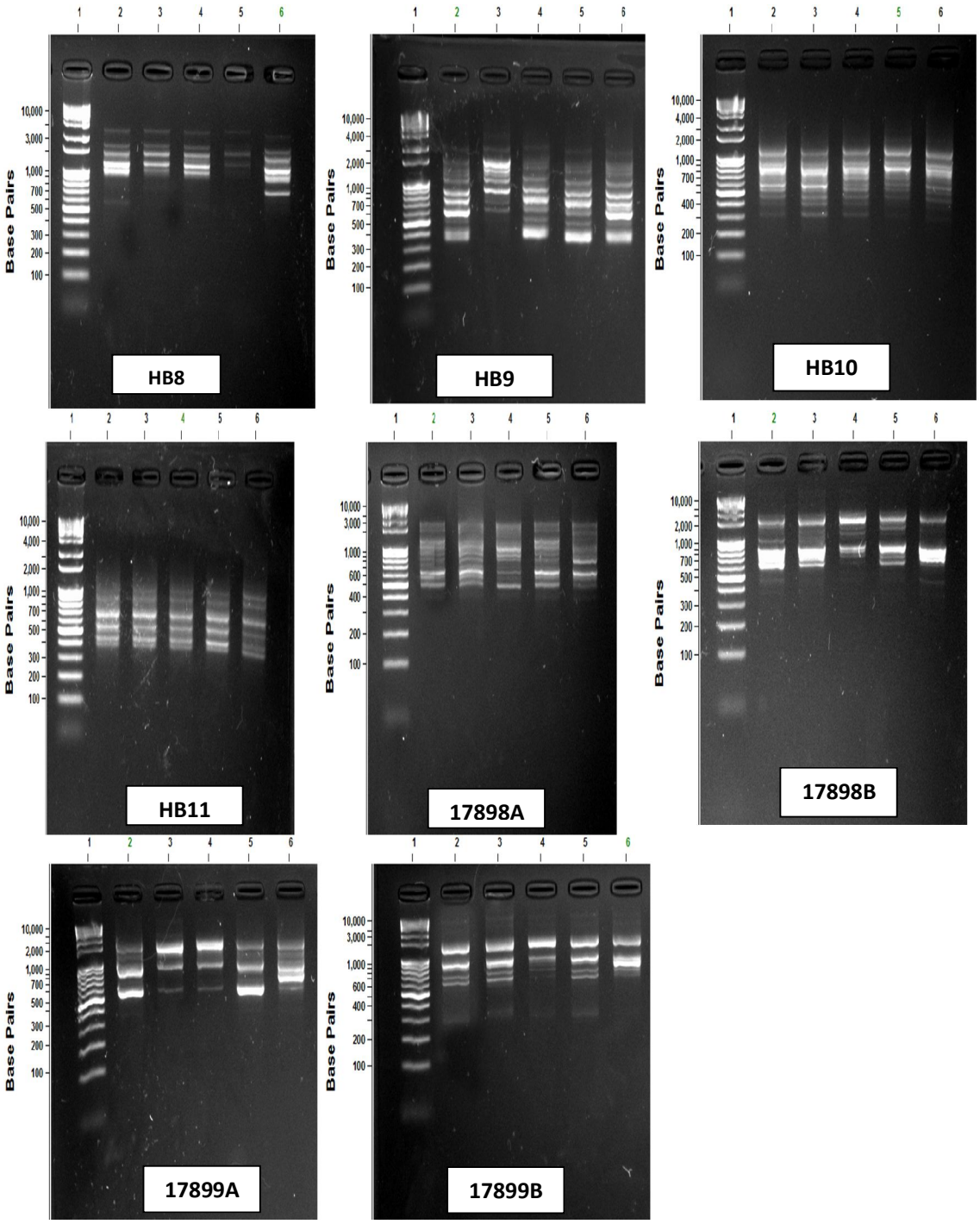

Fig. 2: Amplification of eight ISSR primers used for the five genotypes of pearl millet under salinity. 1- M=100 bp Ladder. 2- PE00056. 3- PE000194. 4- PE00200. 5- PE00463 6- Shanduil-1.

The eight ISSR markers produced different bands with sizes ranging from 100 to 10,000 base pairs. The lowest number of bands was developed by the 17899A primers (5 bands), while the highest Cluster analysis was for relationships among pearl millet genotypes. The dendrogram was estimated by ISSR data located the pearl millet genotypes into two main clusters (Fig. 3). Cluster 1 was separated into three subclusters. The first subcluster included PE00056 and PE000194 genotypes, the second

subcluster included PE00200 genotype only, and the third cluster was included Shanduil 1, while the second main cluster contained PE00463 genotype only. These results agree with those reported by Choudhary *et al.* (2021), who found ISSR marker is suitable for diversity studing and the genetic baise for abiotic stress in pearl millet. Afiah *et al.* (2016) explored ISSR system to differentiate some faba bean genotypes under salt stress. In addition, Animasaun *et al.* (2015) studied ISSRs marker for pearl millet, and their results demonstrated the high genetic diversity give among the genotypes from different origins.

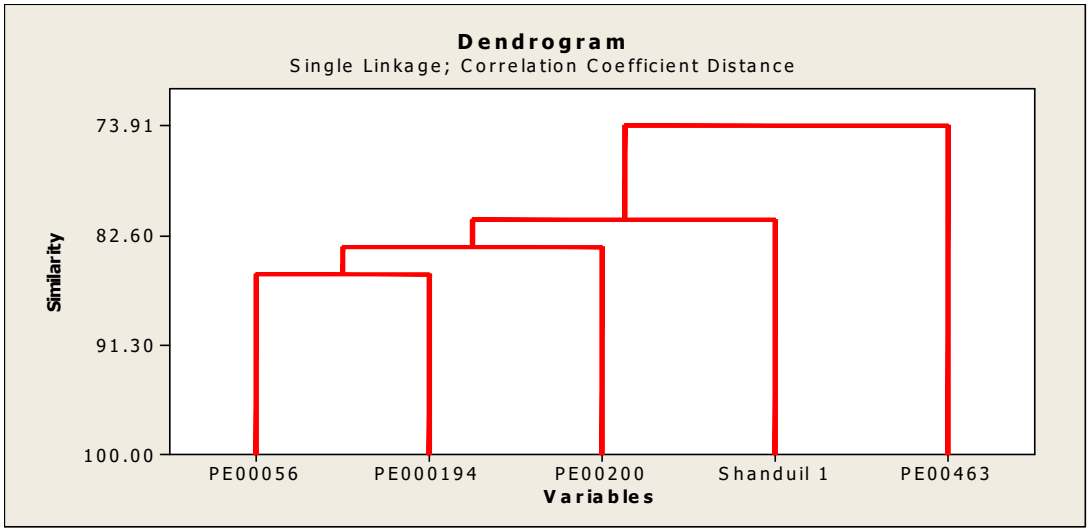

Fig. 3: Linkage dendrogram of five pearl millet genotypes generated by UPGMA cluster analysis based on ISSR markers.

Based on the ISSR results, the similarity relationships between pearl millet genotypes had a range between 0.884 and 0.635 (Table 8) as calculated according to Nei and Li (1979). The highest similarity value was estimated between PE000194 and PE00200 genotypes (0.884), while the lowest was recorded between PE00056 and PE00463 genotypes (0.635). In addition to this

There were other estimates similar to what was obtained. To name a few, there are similar findings by Animasaun *et al.* (2015) and Yazdizadeh *et al.* (2020), who reported that the ISSR molecular marker is an effective marker for assessing genetic diversity. The relationship between most of the genotypes indicated that they had a kinship through a common ancestor. Best of all, the results in Table (8) show the matrix of standardized distance for NEI among the five pearl millet genotypes using ISSR markers. In general, genotypes of PE00056 showed the lowest distance with genotypes PE00200 and PE000194. While the highest distance between PE00056 and PE00463 genotypes was observed between matches.

Table 8: Similarity matrix among five pearl millet genotypes based on ISSR.

Proximity Matrix				
Matrix File Input				
Case	**PE000194**	**PE00200**	**PE00463**	**Shanduil 1**
PE00056	0.871	0.764	0.635	0.733
PE000194	----	0.884	0.703	0.795
PE00200	----	----	0.767	0.818
PE00463	-----	----	-----	0.779
Shanduil-1	-----	-----	----	----

Measurement of genetic distance should be essential for breeding when it is based on a broad range of traits relevant to breeding objectives. Cluster analysis for investigated growth traits, fresh yield, dry forage yield, chemical analysis, and ISSR markers showed diversity among investigated pearl millet

genotypes. It was more comparable to that obtained separately by growth traits, fresh yield, dry forage yield, and chemical analysis. Thus, the five pearl millet genotypes were clustered into two main clusters (Fig. 4). Cluster 1 is separated into two subclusters. The first subcluster included PE00056 and Shanduil-1 genotypes and the second subcluster included PE000194 genotype only, while the second cluster was included PE00200 and PE00463 genotypes. This finding indicated that many lines had a common origin, which envisaged falling into a single cluster. The results of data were supported by Athoni *et al.* (2016) and Singh *et al.* (2017).

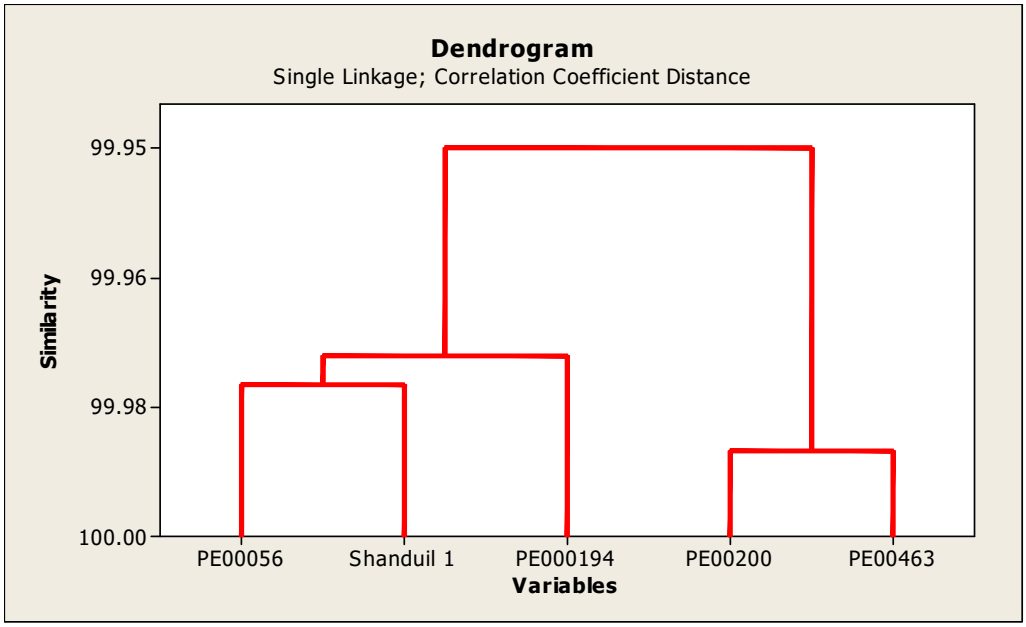

Fig. 4: Linkage dendrogram of five pearl millet genotypes generated by UPGMA cluster analysis based on growth traits, fresh yield, dry forage yield, chemical analysis, and ISSR markers.

3.7. Principal Component Analysis (PCA)

PCA is one of the fundamental analyses by simplification of data. In addition to, PCA of the five pearl millet genotypes under study based on growth traits, fresh yield, dry forage yield, chemical analysis, and ISSR markers to detect superior genotype under salinity stress were placed in different quadrants at extreme ends of the plot (Fig. 5). The analysis has grouped the five genotypes into three different groups that valued for 68.7% of the variation of results. The first and two principal components (PC1 and PC2) were explained 40.4% and 28.3%, respectively. The first quadrant was included PE00056 and PE000194 genotypes, while the second quadrant was contained PE00200 and Shanduil-1 genotypes, and the third quadrant was included PE00463 genotype only. This finding suggests that these genotypes were highly varied and diverse for all the characters. Overall, the PCA analysis under this study shows that phenotypic and molecular markers were useful in genotypes of pearl millet and able to identify a few key traits that accounted for the largest variability. The study confirms with Karunya *et al. (*2021), who were evaluated 25 pearl millet genotypes by PCA and found that some genotypes were positioned further away from biplot origin and cause more variation in traits of the principal components involved than other genotypes.

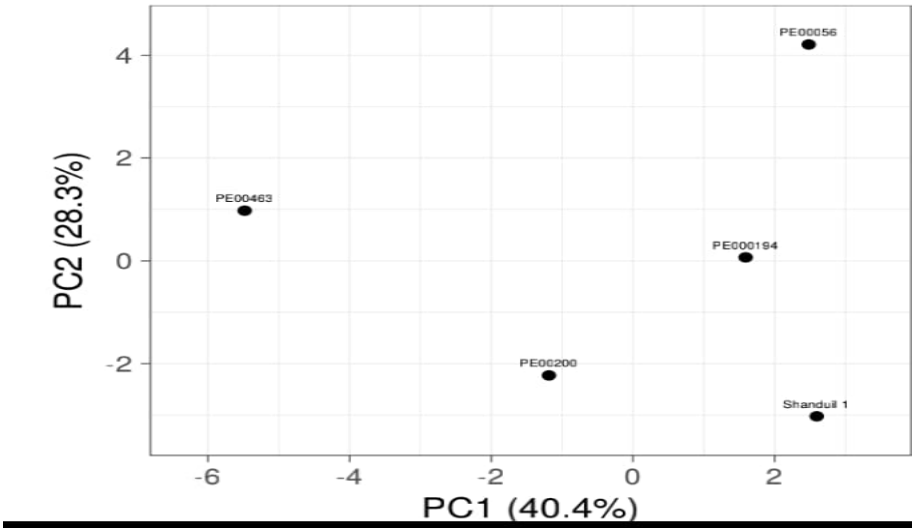

Fig. 5: Biplot representing the distribution of five pearl millet genotypes among PC1 and PC2

3.8. Multivariate Heat map

To provide further evidence heat map analysis were used, and the genotypes were distributed into two main groups. The first group was separated into two sub-groups: the first sub-group containing PE000194 and PE00056 genotypes, and the second sub-group containing only Shanduil-1. Furthermore, group II contains genotypes PE00463 and PE00200 (Fig. 6). In this regard, Animasaun *et al*. (2015) reported that ISSR markers are ideal for genetic diversity analysis and genetic association maps to study tolerance to the harsh conditions of pearl millet.

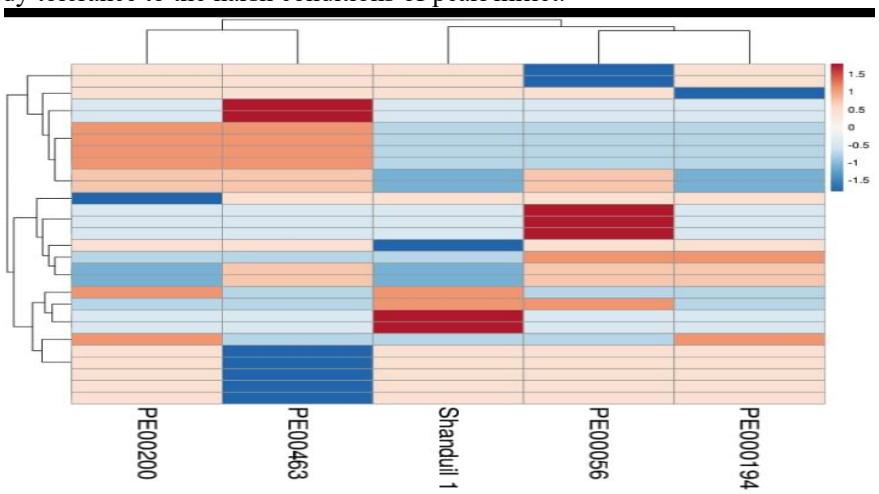

Fig. 6: Multivariate heatmap illustrating the genetic diversity of five pearl millet genotypes based on the eight ISSR markers using the module of a heatmap of R softwwere.

4. Conclusion

Morphological traits and molecular markers analyses were essential tools and complementary for estimating genetic variability among genotypes and used in the breeding programs for improvement. Data appeared significant variation among the studied genotypes of saline stress-tolerant for all different traits in successive seasons, indicating the presence of genetic variability within genotypes to saline stress tolerant. Results suggest that the PE00463 genotype of the pearl millet achieved superior for getting the highest values for growth traits, total fresh and dry yields with better crude fiber and protein compared to the other genotypes followed by the PE00200 genotype. In contrast, the PE000194 genotype recorded the lowest values under soil salinity stress. Generally, both genotypes PE00463 and PE00200 maybe can be featured in the salt tolerance program.

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