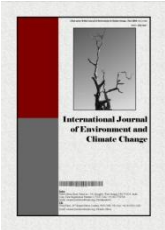

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Molecular Characterization of Drought Tolerant Genotypes of Pearl Millet [*Pennisetum glaucum* (L.) R. Br.] for A_1 Zone

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Authors' contributions

This work was carried out in collaboration among all authors. Author SA designed and executed the study and wrote the whole manuscript. Authors RM, NRG, VK, RCM supported in conducting the experiment. Author CTS edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Pearl millet is a widely grown, climate resilient rainfed cereal crop cultivated on 29 million ha in the arid and semi-arid tropical regions of Asia and Africa accounting for almost half of global millet production. It is useful for minimizing the adverse effect of climate change, hence facilitating income and food security among farming communities. It has deep root system and exhibit climate-resilient features including adaptation to a wide range of ecological conditions, less irrigational requirements, better growth and productivity in low nutrient input conditions, less dependent on synthetic fertilizers and minimum vulnerability to environmental stresses and thus can survive in harsh climatic conditions, less fertile soil under water scarcity. Breeding of drought tolerant varieties and selecting genotypes for better water use efficiency is important in pearl millet to mitigate the changing climatic scenario. In this study, 24 genotypes of pearl millet which are drought tolerant and specific for A_1 zone were characterized using 15 drought specific SSR primers. All the 15 SSRs amplified products of varying sizes ranging between 90-550 bp. A total of 40 alleles were obtained in this study and the number of alleles per locus varied between 2 to 5 with an average of 2.67 alleles. Polymorphic Information Content (PIC) varied from 0.34 to 0.76 with an average of 0.53 PIC value. This study will be useful for developing high yielding, dual purpose cultivars for low rainfall areas i.e. A_1 zone and increasing pearl millet productivity.

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Keywords: Drought tolerance; molecular characterization; pearl millet; SSRs; climate-resilience; abiotic stress.

1. INTRODUCTION

Pearl millet *Pennisetum glaucum* (L.) R. Br.] is the 6th most important cereal crop followed by rice, wheat, maize, barley and sorghum. It is the staple food for around 90 million people and is grown on 29 mha in the arid and semi-arid regions of Asia and Africa which receive rainfall from 150-700 mm [1]. It is the 4th most widely grown crop in India after rice, wheat and maize. Rajasthan, Gujarat, Haryana, Uttar Pradesh and Maharashtra are the major pearl millet growing states contributing 90% of total production in India. Out of this, Rajasthan contributes a maximum of around 4.28 million tonnes followed by Uttar Pradesh (1.30), Haryana (1.08), Gujarat (0.96), Maharashtra (0.67) and Tamil Nadu (0.08). It can survive very well in harsh conditions of drought, high temperature, salinity, lodging and poor soils due to its good adaptability under adverse conditions. Further, it also possess a huge potential for high dry matter production at water deficit and high temperature which makes it a crop of choice for cultivation in arid and semi-arid regions of the world [2]. Pearl millet is very useful as it is a nutritious, climate change ready crop capable of yielding economic returns in marginal conditions where other cereals fail. It is more resilient to extreme conditions of drought and water scarcity and thus can play a significant role in the present situation of changing climatic conditions which are escalating to alarming rate. It is a dual purpose crop and the grain is also used as animal feed for dairy and poultry, alcohol industry, starch industry, processed food in addition to human consumption. It has high nutritional value and is a rich source of energy, carbohydrates, crude fibres, soluble and insoluble fat, proteins (8-19%), ash, dietary fibres (1.2 g/100g), antioxidants, fat (3-8%), vitamins and minerals (2.3mg/100g) like iron, zinc, potassium, copper, magnesium, phosphorous and manganese [3]. In the present scenario of changing climate, it is being affected by various abiotic stresses like drought, salinity, heat etc. and among all these, drought is the most important which limits its production by averting it from expressing its full genetic potential [4,5]. The growing conditions for pearl millet vary from near-optimum to highly drought-prone environments in India. This led to the distribution of various crop growing regions into three zones viz. A_1, A and B. Zone A_1 is comprised of parts of Rajasthan, Gujarat and Haryana which receive <400 mm annual rainfall. Pearl millet is still an important staple crop in A_1 zone and 33 kg per capita per annum is consumed in rural Rajasthan and 28 kg per capita per annum in rural Gujarat and the farmer's income is mainly dependent on this crop in these areas [6]. But, despite the various breeding efforts, there is narrow cultivar diversity in this drought-prone ecology thus leaving very less cultivar choice for the farmers. Farmers in 55% of the pearl millet area (about 4.5 m ha) including A_1 zone are yet to harness the benefits of pearl millet breeding programs in public and private sector. Pearl millet is still a staple cereal in these areas because no other cereal is well adapted or productive under seasonal rainfall of 250 to 300 mm in A_1 zone [7]. Yield in these areas are low and variation in annual production can be extremely high. Under such conditions, the prospects for major increase in production based on introducing drought suitable genotypes in addition to other agronomic practices is needed to increase its productivity.

Conventional approaches are time consuming and need the help of modern biotechnological tools to accelerate millet development programs. Molecular tools and genomic studies have become major approaches these days because they have more potential to improve the efficiency and accuracy of conventional breeding and thus can play a major role in crop improvement programs [8]. In case of drought tolerance, availability of markers tightly linked to tolerant genes will help in identifying plants carrying these genes. Microsatellite markers are considered as the most effective and reliable markers for such studies due to their abundance in the genome, even distribution, easy detection, multi allelic nature, genome specificity, high-throughput, high reproducibility and co-dominant behaviour [9,10]. Therefore, the use of SSR markers is a precious approach for molecular characterization among pearl millet genotypes. Hence, the present investigation was undertaken to identify and characterize the genotypes suitable for drought prone areas of A_1 zone.

2. MATERIALS AND METHODS

2.1 Plant Material

Plant material comprised of a total of 24 genotypes including popular pearl millet hybrids and varieties along with drought/heat tolerant

lines suitable for A_1 zone developed under Indian Council of Agricultural Research-All India Coordinated Research Program on Pearl Millet, Jodhpur, India (Table 1). Twelve days old, tender and healthy leaves of plants grown in field conditions were collected in labeled self-sealing polyethylene bags and stored at -20°C until use. The molecular marker analysis was performed at PC Unit, ICAR-AICRP on Pearl millet, Jodhpur during 2020-21.

2.2 Genomic DNA Isolation and Quantification

Genomic DNA was extracted from young and fresh leaves of 12 days old plantlets of 24 genotypes using cetyl trimethyl ammonium bromide (CTAB) method along with some modifications as reported in our other study [11]. 2-3 leaves from each genotype were ground in a pestle and mortar without liquid nitrogen and the mixture was transferred into 2 ml eppendorf tubes and 1200 µl of CTAB extraction buffer was added to them. The samples were incubated for 2 hrs at 65°C in a water bath with intermittent mixing by gently inverting the tubes. They were centrifuged for 10 mins at 12,000 rpm and the supernatant was collected into fresh tubes. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently followed by centrifugation at 12,000 rpm for 10 mins. The supernatant was carefully collected again into fresh centrifuge tubes. Five µl of RNase A (10 mg/ml) was added to each tube and incubated at 40°C for 45 mins in a water bath. Later, equal volume of CI (24:1) was added to the tubes followed by centrifugation at 12,000 rpm for 10 mins. The aqueous layer was collected again in fresh tubes and DNA was precipitated by adding 1/10 volume of 3M sodium acetate and equal volume of chilled isopropanol. The samples were stored at -20°C for overnight and next day centrifuged at 12,000 rpm for 10 mins. The supernatant was discarded and DNA pellet was washed twice with 70% ethanol. The pellet was air dried and dissolved in 80 µl TE buffer and stored at -20°C for future use. The extracted DNA was analyzed qualitatively on 0.8% agarose gel and diluted to a final concentration of 10 ng/µl for PCR reactions.

2.3 PCR Analysis

PCR was carried out in a volume of 10 µl amplification reactions using 15 drought specific SSR primers in a 96-well thermal cycler (Agilent Technologies) as described by Ambawat et al. [10]. Master mix consisted of 10 µl reaction mixture having 1X Buffer, 200 mM each dNTP, 0.4 µM 10-mer primer, 1 unit Taq DNA polymerase (Bangalore GeNei, India) and 10 ng of DNA. This reaction mixture was allowed to undergo initial denaturation at 94°C for 5 min followed by 35 cycles of 30s at 94°C for denaturation, 30 s at 58°C for annealing and 1 min at 72°C for primer extension. Lastly, 1 cycle of final extension was carried out for 10 mins at 72°C followed by hold at 4°C. The amplified products were analyzed on 3.5% agarose gel which was prepared by dissolving 5.25 g of agarose in 15 ml of 10X TBE buffer and 135 ml of sterile distilled water. It was melted in a microwave oven and added 5 µl of ethidium bromide after cooling to 65°C and casted in a sealed gel tray having a comb. The amplified products were loaded in the gel and run at 150 V for 2 hrs. After separation, the gel was visualized on a Gel Doc system, photographed and documented.

3. RESULTS AND DISCUSSION

3.1 Molecular Characterization and SSR Analysis

Till date, a total of 185 hybrids and 62 varieties have been identified and released for cultivation in different agro ecological zones of India through ICAR-All India Coordinated Research Project on Pearl millet which helped to enhance the production and productivity of pearl millet to a large extent [12]. From 1950 to 2018, public and private sector has released 58 hybrids for A zone (Rajasthan, Haryana, Gujarat, Madhya Pradesh, Uttar Pradesh, Punjab and Delhi), 36 for B zone (Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu), 39 hybrids for all India (A&B zones) but only 15 hybrids for A_1 zone (areas of Rajasthan, Haryana and Gujarat having <400 mm annual rainfall) and only 11 hybrids for summer areas, while western parts of A_1 zone are still cultivating landraces as no hybrid (except HHB 67 Imp.) has been able to fetch for these harshest environment [6]. Various efforts have been made to screen genotypes specifically for A_1 zone against abiotic stresses like drought and heat which can be useful for developing hybrids/varieties for this specific zone [13-15]. But these reports mainly include morphological and physiological approaches of characterization which are time-consuming, inefficient, expensive, restricted to a few characteristics, influenced by environmental effects [16]. Contrary to it, the present study is based on molecular

characterization of genotypes for A_1 zone which was carried out using the most efficient and preferred molecular markers i.e. SSRs. The findings of the study can indubitably be used further for developing high yielding cultivars for low rainfall areas i.e. A_1 zone and can be anticipated to improve the efficiency of breeding for improved drought tolerance and increase the pearl millet productivity.

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Table 1. List of genotypes used for molecular characterization

S. No.	Name of Hybrid/Variety	Year	Organization	Salient features
1.	RHB 234	2019	ICAR-AICRP on Pearl millet, RARI, Jaipur	Medium maturing, brown anther colour, complete excertion, greyish seed, resistant to major diseases & insect pest
2.	RHB233	2019	ICAR-AICRP on Pearl millet, RARI, Jaipur	Medium maturing, yellow anther colour, complete excertion, greyish seed, resistant to major diseases & insect pest
3.	RHB223	2018	ICAR-AICRP on Pearl millet, RARI, Jaipur	Early maturing, brown anthers, long brown bristles, resistant to downy mildew, blast and smut, resistant to shoot fly, stem borer and grey weevil, tolerant to stress
4.	RHB177	2011	AICPMIP, RARI, Jaipur	Early maturing, medium tall, cylindrical bristled earheads, resistant to downy mildew, light yellow anthers
5.	MPMH17	2013	AICPMIP, Jodhpur	Medium maturing, medium height, yellow anther colour, compact lanceolate earheads with bristles, resistant to downy mildew, grey brown seeds
6.	MPMH21	2016	ICAR-AICRP on Pearl millet, Jodhpur	Early maturing, brown anthers, lanceolate compact ear heads, grey brown hexagonal grains, resistant to downy mildew, blast and smut
7.	BHB1202	2018	ICAR-AICRP on Pearl millet, SKRAU, Bikaner	Early maturing, compact conical earhead, yellow brown globular shaped grains, yellow brown anther color, highly resistant to downy mildew, blast and major pests
8.	AHB 1269	2019	NARP, Aurangabad	Medium maturing, long cylindrical compact panicles, high Fe content, resistant to downy mildew and stem borer
9.	DHBH 1397	2019	ICAR-AICRP on Pearl millet, BRS, MPKV, Dhule	Medium maturing, dual purpose hybrid, globular shaped grey colour seed, highly resistant to downy mildew and blast
10.	Pusa Composite 443	2011	AICPMIP, IARI, New Delhi	Early maturity, medium tall, rod shaped earheads with bold grain
11.	HHB272	2016	ICAR-AICRP on Pearl millet, CCS HAU, Hisar	Early maturing, brown anthers, lanceolate compact ear heads, grey globular grains, resistant to blast
12.	HHB234	2013	AICPMIP, CCS HAU, Hisar	Early maturing, candle shaped earheads with small bristles, medium seed size and tolerant to downy mildew
13.	JKBH 1326	2019	JK Agri Genetics, Hyderabad	Medium maturing, medium thick, very long, conical and compact head, clear exertion with small to medium size pale yellow grain, highly resistant to downy mildew, rust, smut, ergot and blast
14.	PB1705	2018	Bayer Bio	Medium maturing, grey color grain with

S. No.	Name of Hybrid/Variety	Year	Organization	Salient features
			Science Pvt. Ltd., Hyderabad	bold size, resistant to DM, blast, rust, smut & ergot, tolerant to lodging, tolerant to shoot fly and stem borer
15.	PB 1852	2019	Bayer Bio Science Pvt. Ltd., Hyderabad	Medium maturing, grey colour grain with bold size, lodging tolerant, responsive to fertilizers, resistant to DM and blast, tolerant to moisture stress
16.	NBH4903	2018	Nuziveedu Seeds Pvt. Ltd., Hyderabad	Late maturing, medium plant height with long exerted compact panicles, medium bold grains, non lodging ,non shattering, resistant to drought
17.	HHB226	2011	AICPMIP, CCS HAU, Hisar	Medium maturing, medium height, dark green leaves, candle shaped bristled earheads, resistant to downy mildew
18.	GHB538	2009	AICPMIP, MRS, Jamnagar	Early maturity, highly resistant to moisture stresses, resistance to downy mildew and lodging
19.	Improved HHB 67	2009	AICPMIP,CCS HAU, Hisar	Extra early maturity, highly resistant to moisture stresses, resistant to downy mildew. The first commercial cultivars developed using marker-assisted selection in India
20.	GHB719	2007	AICRP on Pearl millet, MRS, Jamnagar	70-75 days maturity, fully exerted conical shaped, compact and bristled earheads, globular, medium in size, grey coloured grains, tolerant to drought
21.	CZP9802	2003	CAZRI, Jodhpur	70-72 days, medium tall, good tillering, thin stem, narrow leaves, thin candle-shaped earheads, yellowish grains of medium size, drought tolerant, very high stover of good quality
22.	MIR524	-	Agriculture University, Jodhpur	Drought tolerant line developed for A_1 zone
23.	BIB448	-	SKRAU, Bikaner	Drought tolerant line developed for A_1 one
24.	BIB445	-	SKRAU, Bikaner	Drought tolerant line developed for A_1 one

PCR amplification and molecular characterization among 24 pearl millet genotypes was carried out using 15 SSR primers specifically reported for drought [17]. All the 15 SSRs amplified products of varying sizes ranging between 90-550 bp (Figs. 1, 2). A total of 40 alleles were obtained in this study and the number of alleles per locus varied between 2 to 5 with an average of 2.67 alleles. Similar kinds of reports were observed by [10,18,19]. But, they were relatively lower than 2-18 alleles (6.8 alleles per locus), 4.62 alleles per primer and 12.5 alleles per locus as observed by different investigators in other studies [20-22]. Polymorphic Information Content (PIC) varied from 0.34 to 0.76 with an average of 0.53 PIC value (Table 2). The highest PIC value was observed for the marker PSMP2066 (0.76) followed by PSMP2077 (0.74), CTM21 (0.67) and PSMP2072 (0.65) while the lowest was for the marker IPES0218 (0.34) and IPES0117 (0.38). Thus, PSMP2066 is the most informative and best marker for characterization followed by PSMP2077, CTM21 and PSMP2072 markers while maker IPES0218 was reported to be the least powerful marker. PIC values ranging from 0.02 to 0.97 were reported by many researchers [21,23-26]. An average PIC value of 0.53 observed in the present study is similar to 0.55, 0.56 and 0.58 reported by other investigators [10,21,27], respectively. PIC values can range between 0 and 0.5 because of bi-allelic nature of SNPs while it can go above 0.5 in case of SSRs due to their mutli-allelic nature as reported by other researchers [10,17,23]. Thus, SSR markers are the most preferred and efficient markers due to their ability to detect multiallelic loci, easy to

use, co-dominance, higher reproducibility, high polymorphism with vast ability to differentiate the genotypes [28]. Similar kind of results regarding effectiveness of SSR markers in molecular characterization have been also reported in other studies [9,10,19,20,21,23,27,29,30].

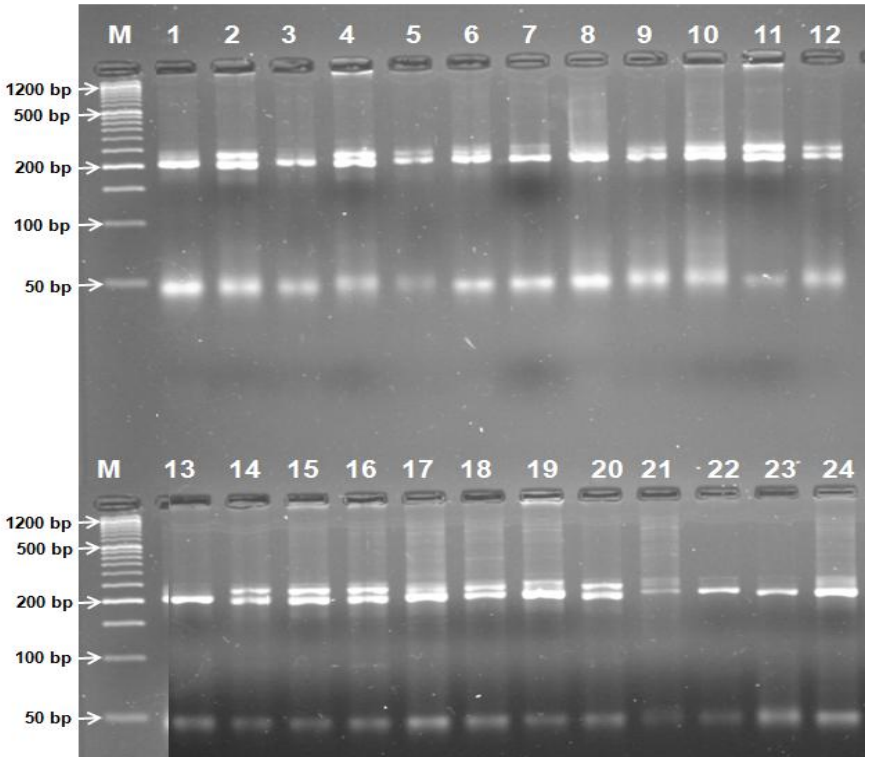

Fig. 1. Agarose gel showing amplification profiles of pearl millet hybrid/varieties using the primer PSMP 2206. Lane M-50 bp ladder, Lane 1-24 pearl millet genotypes

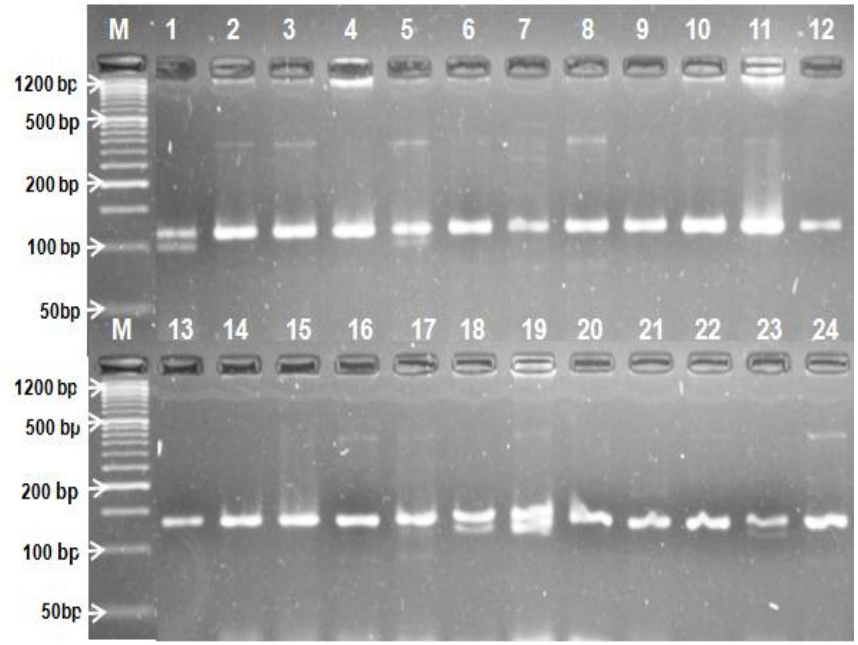

Fig. 2. Agarose gel showing amplification profiles of pearl millet hybrid/varieties using the primer PSMP 3056. Lane M-50 bp ladder, Lane 1-24 pearl millet genotypes

Table 2. List of SSR primers showing polymorphism

S. No.	Oligo Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product range	No. of alleles amplified	PIC value
1.	PSMP2237	TGGCCTTGGCCTTTCCACGCTT	CAATCAGTCCGTAGTCCACACCCCA	250-280	2	0.48
2.	CTM3	GTCCATCGTCGCCGACGAA	GGATTTGCTAGTTGTGGGCT	250-450	3	0.56
3.	PSMP2072	GAAATCTACACAAGGGTCTCCA	GTACGGAGCAATGACATCTGAA	150-350	3	0.65
4.	PSMP2066	ATATTAGAGCATTGCATCGC	GCATAGCAGCATACAGCAGCAA	190-550	4	0.76
5.	ICMP3056	ACGGAGCTACGGTTGGAATA	CACAAGGGACCCCACGATA	140-400	3	0.49
6.	PSMP2206	AGAAGAAGAGGGGGTAAGAAGGAG	AGCAACATCCGTAGAGGTAGAAG	200-210	2	0.43
7.	CTM21	ATGCCTCCCACCCCACGTCG	CGTCGCACTAGCCACAGTCA	90-110	3	0.67
8.	PSMP2059	GGGGAGATGAGAAAACACAATCAC	TCGAGAGAGGAACCTGATCCTAA	110-275	3	0.58
9.	ICMP3063	TCCGGTAGAGACCGTAATGG	GGCACTCCCTAGCAAAATGA	180-200	2	0.50
10.	IPES0236	GGCCAGCTCGCCTAGAT	AGATCCACCGCCTAATGAAA	210-250	2	0.50
11.	IPES0152	GATACGAAGGGAAGCACAGC	TGTGTGGTAAGCTGCTGGAG	105-120	2	0.48
12.	PSMP2077	GCCAATATTATTCCCAAGTGAACA	CTCTTGGTTGCATATCTTTCTTTT	100-300	5	0.74
13.	PSMP2078	CATGCCCATGACAGTATCTTAAT	ACTGTTCGGTTCCAAAATACTT	150-160	2	0.42
14.	IPES0117	TTATTATTCGGTCATCACAGCG	TCCAAAACACAATTCCACCC	100-120	2	0.38
15.	IPES0218	CCTGGGAACACAAAACCAGA	CCAGGTCCATGTCCTTGACT	250-260	2	0.34

4. CONCLUSION

Most of breeding programs fail to deliver products for A_1 zone mainly because this zone has vast variation in microclimate (day & night temperature and humidity) & soil apart from rainfall which requires proper quantification. Hence, there is a high need to screen genotypes which are drought tolerant and can survive in harsh conditions of A_1 zone to develop good cultivars under breeding programs. Such cultivars can survive well under changing climatic scenario. This study will be useful and can be exploited for developing high yielding cultivars for low rainfall areas i.e. A_1 zone. The findings in this study can be expected to improve the efficiency of breeding for improved drought tolerance and increase the pearl millet productivity. They can also prove to be an excellent genomic resource for isolation of candidate genes responsible for tolerance to situation arising out of global warming and climate change for accelerating further genetic improvement of other crops.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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