

Genetic diversity assessment of pearl millet novel male sterile lines based on DNA polymorphism

S K Singh*, V K Manga, Aravind Kumar Jukanti and Rakesh Pathak
ICAR-Central Arid Zone Research Institute, Jodhpur 342 003, India

Received 14 September 2015; revised 19 May 2016; accepted 7 June 2016

Lack of breakthrough in the yield is demanding drought tolerance and adaptation to the harsh arid environment by the released hybrids of pearl millet [*Pennisetum glaucum* (L.) R.Br.]. Fertility restoration patterns of F₁ hybrids developed from crosses between male sterile (MS) lines possessing different cytoplasmic MS sources and a genetically diverse set of inbreds were exploited to circumvent this problem. In this paper, we report the extent of genetic diversity and relationship among 20 newly developed MS lines in *P. glaucum* using nuclear ribosomal DNA and RAPD polymorphism. High level of polymorphism (82.7%) among MS lines of *P. glaucum* by potential RAPD markers and with greater proportion of total genetic variation within population (87%) rather than among population (13%) revealed rich genetic diversity among the MS lines developed. Total gene diversity exhibited high levels of variability ($H_T=0.2337$) and coefficient of gene differentiation ($G_{ST}=0.4651$). Phylogram based on multiple sequences further delineated all the 20 MS lines with significant bootstrap values. SNPs at 16 places in ITS-1 are clear indication of phylogenetic utility of ITS-1 region, in particular, in developing bar code of pearl millet germplasm. The MS line CZMS-21 recorded with desirable traits like significantly higher grain yield and the maximum panicle harvest index was identified as a potential source of A4 cytoplasm for the development of stable single cross hybrid for arid environment. The genetic diversity among these MS lines with adaptation to arid environment shall be of significance in developing region specific hybrids.

Keywords: nrDNA diversity, genetic diversity, male sterile lines, *Pennisetum glaucum*, RAPD

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] is the world's most hardy, warm season, coarse cereal cultivated for grain, stover and green fodder mainly in arid and semi-arid regions of Africa and Asia¹. India is the largest single producer of pearl millet occupying an area of 9.3 M ha with a production of 8.3 M tones, contributing 42% of production in the world². It is an important coarse cereal crop in western India and occupies about 38% of the total cereal crop area in the region. Mainly five states (Rajasthan, Maharashtra, Gujarat, Uttar Pradesh & Haryana) account for nearly 95% of the pearl millet cultivation area. With the advent of hybrids, the yield of pearl millet increased substantially in other states, while it was unsatisfactory in Rajasthan due to lack of desired adaptability and characteristics required for this region³. Under harsh conditions of arid zone, some landraces are also reported for out-yielding hybrids⁴.

For successful cultivation of pearl millet hybrids in the arid western Rajasthan, it is important that the

hybrids should be developed using parents that are adapted to the harsh climatic conditions of this region. Such hybrids would lead to better adaptation and performance, besides providing cultivar diversity, giving more choice to farmers. Hybrid vigour has been extensively utilized in pearl millet for increasing pearl millet productivity by utilizing male sterility for large scale production of hybrid seed. A high degree of heterosis in F₁ hybrids, exceeding high yielding parental lines, up to 425%, have been realized⁵. Nevertheless, this increase in productivity has been primarily restricted to areas with assured rainfall or irrigation and fertilizers⁶.

ICAR-Central Arid Zone Research Institute (CAZRI), Jodhpur, India has a strong restorer parent development programme^{7,8} and is being utilized in crossing with male sterile (MS) lines from other sources like International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru (Telangana), India, to develop hybrids. Majority of the MS lines from other sources lacked adaptability to the harsh climatic conditions of arid region. Hence, a strong need was felt to develop pearl millet MS lines that are well adapted to this region leading to their

*Author for correspondence:
sksingh1111@hotmail.com

successful cultivation with increased productivity. Keeping this in mind, a targeted MS development programme was initiated at ICAR-CAZRI, Jodhpur in 2002. From B composites and segregating populations of B $\times$ B crosses, promising B progenies were carried forward by pedigree selection to develop maintainer inbred lines, which were crossed with other MS lines as source of A₁ and A₄ cytoplasm, followed by backcrossing to develop a set of new MS lines⁹.

Randomly amplified polymorphic DNA (RAPD) is a cost effective and efficient method for genotype identification, pedigree analysis and genome mapping¹⁰. RAPD markers offer advantages like higher frequency of polymorphism, rapidity, technical simplicity, requirement of few nano grams (ng) of DNA, no requirement of prior information of the DNA sequence and feasibility of automation. It allows the analysis of individual and large number of markers in relatively short time, as only a few primers allow the generation of sufficient data to obtain a robust estimate of diversity index, and have allowed the resolution of complex taxonomic relationships. This technique has recently been used on a number of crop plants, such as, *Vigna radiata*¹¹, *Cyamopsis tetragonoloba*¹² and *Pennisetum glaucum*¹³.

Currently, nuclear ribosomal (nr) internal transcribed spacer (ITS), encompassing highly conserved 5.8S rRNA gene, is considered as one of the most useful phylogenetic marker due to less functional constraints and comparatively higher evolutionary changes¹⁴. It evolves relatively quickly and can be useful in determining inter-species¹⁵ and sometimes intra-species relationships¹⁶. The rate and patterns of ITS sequence mutation are typically appropriate for resolving relationships among species and genera¹⁷. Although thousands of copies of the ITS exist in Angiosperm genomes, they are generally homogenized by concerted evolution and thus can be treated as a single locus.

The region of 5.8S rRNA gene was shown to contain considerable phylogenetic information¹⁸. The ITS polymorphism might occur at a genus, species or individual level, making it useful for phylogenetic, evolutionary and bio-geographical diversity studies¹⁹. ITS sequences have been widely used in several species for phylogenetic inferences.

The present study envisages: (i) development of new MS lines to add to the genetic diversity of existing MS lines as well as hybrids; (ii) field evaluation of these novel MS lines under rainfed arid

environment, and (iii) genetic diversity analysis of new MS lines using RAPD and nrDNA analysis.

Materials and Methods

Breeding Strategy

New MS lines (numbering 20) were developed at ICAR-CAZRI, Jodhpur, India by crossing maintainers developed by selection from a B composite developed jointly by ICAR-CAZRI and ICRISAT under a collaborative project, and from F₂ populations of 18 selected crosses from a diallel cross. Promising S₆ progenies from the composite and F₆ progenies from segregating populations were then crossed with MS lines having ICMA 95111 and ICMA 94555, the source of A₁ cytoplasm, and ICMA 95111, a source of A₄ cytoplasm, and then backcrossed with their respective B lines to generate new MS lines. Pedigree of the MS lines is given in Table 1.

Field Experimentation

These MS lines were planted during the rainy season in the year 2013 in a randomized complete block design with three replications at the ICAR-CAZRI, Jodhpur (26° 18' N, 73° 01' E). The lines were planted in the month of July and harvested in October. During the cropping period, crop received total precipitation of 389.9 mm. Of this, 244.8 mm rainfall was received in the months of July and August, the remaining 145.1 mm rainfall was received in the last week of September, when crop was almost mature. Each entry was planted in two rows of 4 m length with row spacing of 60 cm and plant to plant spacing 15 cm. The experiment was conducted in the loamy sand with applied fertilizer of 40 kg nitrogen/ha (50% basal and rest as top dressing) and 20 kg phosphorous/ha (basal dose). The standard cultural and agronomic practices were followed that included thinning and weeding at 15 d after sowing.

Field Observations

Data were recorded on 5 plants for plant height, days to 50% flowering, effective tillers/plant, ear head length (cm), ear head girth (cm), ear head wt (g)/plot, grain yield (g)/plot and 500 grain wt (g). The above data were used to compute panicle harvest index.

RAPD Analysis

A set of 18 decamer random primers of OPA, OPB and OPP series (Operon Technologies) was used for initial screening of all the MS lines of *P. glaucum*. Finally, the data of 12 RAPD primers exhibiting

Table 1 — Pedigree of MS lines of *P. glaucum* developed at ICAR-CAZRI, Jodhpur

No.	Male sterile lines	Pedigree
1	CZMS 0001A	ICMA 95111 source of A ₁ cytoplasm backcrossed to CZMS 0001B = ICMB 95111 × [843B × EEBC-S1-407]12-2-B-B)7-2-B-B-B-B-1-1-1-1
2	CZMS 0002A	ICMA 95111 source of A ₁ cytoplasm backcrossed to CZMS 0002B = IC CZBC C0-96-5-B-1-2-1-1
3	CZMS 0003A	ICMA 95111 source of A ₁ cytoplasm backcrossed to CZMS 0003B = ICMB 95111 × [843B × EEBC-S1-407]12-2-B-B)7-2-B-B-B-B-2-1-1-1
4	CZMS 0004A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0004B = IC CZBC C0-60-3-B-2-1-1-1-1
5	CZMS 0005A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0005B = IC CZBC C0-60-3-B-2-1-2-1-1
6	CZMS 0006A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0006B = IC CZBC C0-96-5-B-1-2-2-1
7	CZMS 0007A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0007B = IC CZBC C0-19-5-B-1-1-1-1-1
8	CZMS 0008A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0008B = [ICMB 94555 × ICMB 96333]-4-B-1-1-1-1
9	CZMS 0009A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0009B = [ICMB 94555 × ICMB 96333]-4-B-2-1-1-1
10	CZMS 0011A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0011B = IC CZBC C0-60-3-B-2-1-2-2-1
11	CZMS 0012A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0012B = IC CZBC C0-96-5-B-1-3-1-1
12	CZMS 0013A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0013B = ICMB 95111 × [843B × EEBC-S1-407]12-2-B-B)7-2-B-B-B-B-2-1-1-2
13	CZMS 0014A	ICMA 94555 source of A ₁ cytoplasm backcrossed to CZMS 0014B = ICMB 95111 × [843B × EEBC-S1-407]12-2-B-B)7-2-B-B-B-B-3-1-1-1
14	CZMS 0015A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0015B = IC CZBC C0-60-3-B-2-1-2-2-2
15	CZMS 0017A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0017B = IC CZBC C0-60-3-B-2-1-3-1-1
16	CZMS 0018A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0018B = IC CZBC C0-60-3-B-4-1-1-1-1
17	CZMS 0019A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0019B = IC CZBC C0-60-3-B-2-1-5-1-1
18	CZMS 0020A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0020B = IC CZBC C0-60-3-B-2-6-1-1-1
19	CZMS 0021A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0021B = IC CZBC C0-60-3-B-2-5-1-1-1
20	CZMS 0022A	ICMA 97555 source of A ₄ cytoplasm backcrossed to CZMS 0022B = IC CZBC C0-60-3-B-2-4-2-1-1

consistent and reproducible results was used for analysis. Each amplification was performed in a total reaction mixture of 25 µL containing: decamer primer, 1 µL (50 pmol/µL); dNTP mix, 2 µL (2 mM each of dATP, dGTP, dCTP and dTTP from MBI, Fermentas); MgCl₂, 1 µL (25 mM, MBI, Fermentas); Taq DNA polymerase, 0.4 µL (5 U/µL, Sigma Chem); 10× PCR buffer, 2.5 µL (Sigma Chem) and 4 µL of genomic DNA (50 ng/µL) in dH₂O. PCR amplifications were performed in a gradient thermal cycler (Corbett Research, USA) with initial denaturation step of 94°C for 3 min, followed by 38 cycles of 94°C for 40 sec, 50°C for 40 sec and 72°C for 2 min, and final elongation at 72°C for 10 min.

Amplicons were separated on 1.6% agarose gel (Sigma) pre-stained with ethidium bromide solution using 1× TAE buffer. The gel was run for 3 h at 50 V and the RAPD amplicon profiles were recorded using Syngene Gel Documentation System with Genesnap software. The size of amplified fragments was determined using 1 kb ladder (MBI Fermentas). All RAPD reactions were performed twice to test the reproducibility of the amplicon profile.

ITS Amplification

ITS-1 and ITS-4 universal primers developed by White *et al*²⁰ were used for ITS amplification. Each PCR amplification was performed in a total volume of 50 µL containing: 1 U Taq DNA polymerase (Sigma Chem), 2.5 mM MgCl₂, 160 µM dNTP mix (MBI Fermentas), 50 pmol of each of the ITS-1 and ITS-4 primers (Bangalore Genei), and 50 ng genomic DNA in dH₂O. The reactions were performed in a thermal cycler with the following conditions: 1 min denaturation at 95°C, 30 sec annealing at 50°C, 90 sec elongation at 72°C, for 34 cycles with a final elongation step of 72°C for 10 min.

Amplified ITS regions were sequenced employing ABI Prism DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) using ITS-1 and ITS-4 primers, separately for DNA labeling by the BigDye terminator method. The sequenced data obtained from the ITS-4 primer were inverted using Gene Doc software and clubbed with the sequence data obtained with the ITS-1 primer, to obtain the complete sequence of the ITS region. Comparison of nucleotide sequences was performed using the Basic Local

Alignment Search Tool (BLAST) available at the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). Molecular characterization was done on the basis of similarity with the best aligned sequence of BLAST search.

Molecular Analysis of RAPD

The RAPD amplification products were scored as present (1) and absent (0) of scorable loci for each primer isolate combination. Molecular data were entered into a binomial matrix and were used to determine Jaccard's similarity coefficient with NTSYS-pc software. Most informative primers were selected based on high polymorphism information content (PIC) value of individual primers.

$$PIC = \sum_{i=1}^n 2F(1-F)$$

Where F is frequency of presence of marker band, *i* is discrimination rate (DR) estimated to test the efficacy of individual primers in distinguishing the isolates, employing the formula DR = number of pairs of isolates differentiated/total number of pairs.

To perform molecular analysis, all the 20 MS lines of pearl millet were divided into six populations based on the pedigree from which they were developed. Accordingly the pop-1 (CZBC CO-60) included CZMS-4A, 5A, 11A, 15A, 17A, 18A, 19A, 20A, 21A and 22A; pop-2 (CZBC CO-96) included CZMS-2A, 6A and 12A; pop-3 (ICMB 95111 × [843B × EEBC-S1-407]) included CZMS-1A, 3A, 13A and 14A; pop-4 (ICMB 94555 × ICMB 96333) included CZMS-8A and 9A; and pop-5 (CZBC CO-19) represented by CZMS-7A. Principal Coordinate Analysis (PCA) via covariance matrix was calculated using GenALEX 6 software²¹. On the other hand, diversity in the frequency of fragment size of RAPD patterns was apportioned within and among CMS (cytoplasmic MS) lines using Shannon's information index (*i*) and gene diversity index (*h*), following Nei using PopGen 32 programme.

The total genetic diversity (H_T), genetic diversity within each population (H_S) and coefficient of gene differentiation (G_{ST}) were determined according to Nei²². Nei's unbiased genetic distance and genetic identity were calculated for each population pair using the program. These indices were measured by POPGENE version 1.31 software. To examine the genetic relationship among populations, a dendrogram based on unbiased genetic distance was generated using UPGMA of NTSYS-pc version 2.02c. The gene flow estimates²³ (N_m) among these populations were

computed as $N_m \frac{1}{4} (1/G_{ST}-1)/4$. An analysis of molecular variance (AMOVA) was performed to calculate the hierarchical apportionment of variation.

Sequence Analysis

Nucleotide sequence comparisons with that of available reference sequences were performed using the BLAST search of NCBI. The multiple sequence alignment of the ITS region (ITS-1, 5.8S r-RNA gene & ITS-2) representing 20 MS lines of pearl millet was performed using CLUSTAL X 1.83 software depicting bootstrap values using NJ plot software based on single nucleotide polymorphisms (SNPs), small insertions/deletions (INDELs). The phylogenetic relationship among MS lines was estimated after the construction of a phylogram based on multiple sequence alignment of rDNA ITS sequences with the Tree View software.

Results and Discussion

Perusal of morphological characterization data revealed statistically significant variations among 20 MS lines developed for arid environment of Rajasthan (Table 2). Days to flower varied 40 (CZMS-9A) to 53 d (CZMS-4A, 13A, 22A), plant height 81 (CZMS-17A) to 179 cm (CZMS-21A), ear head length 11 (CZMS-6A) to 20 cm (CZMS-18A), and ear head girth 1.2 (CZMS-9A) to 2 cm (CZMS-1A). Effective tillers 2 to 6 in numbers, ear head numbers 51 (CZMS-12A) to 138 (CZMS-9A)/plot, and ear head wt varied the least 522 g in CZMS-12A to the maximum of 2481 g in CZMS-21A. The grain yield also varied significantly from the minimum of 197 g (CZMS-12A) to the maximum of 1855 g (CZMS-21A). Variations in 500 grain wt was also recorded ranging 3 (CZMS-2A) to 4.3 g (CZMS-9A). The panicle harvest index significantly varied 0.42 (CZMS-4A) to 0.75 (CZMS-21A).

A fairly good estimate of ear head length and ear head girth can be ascertained in the pictorial view of ear heads of CMS lines studied (Fig. 1). The observations on morphological characterization substantiated by pictorial ear head diversity is clear indicative of substantial diversity among 20 MS lines of *P. glaucum*. Grain yield is a complex character and improvement in grain yield contributing traits, such as, number of tillers/plant, ear length, ear girth, number of grains/ear, 1000-grain wt etc, have also been reported to improve grain yield in *P. glaucum*^{5,24}. The MS line CZMS-21 was recorded with desirable traits, such as, early flowering at 44 d,

the maximum plant height 179 cm, 18 effective tillers, 1.9 cm ear girth, 2481 g ear head wt/plot and significantly higher grain yield of 1855 g with the maximum panicle harvest index of 0.75 had been identified as a potential source for development of single cross hybrid for arid environment. Most of these traits had also been reported to have positive association with grain yield²⁴.

Twelve RAPD primers detected intra-specific variation generating scorable amplicons, reproducible patterns and generated 121 marker bands (Table 3). Among these, 103 markers were polymorphic amounting to 82.7% polymorphism and exhibited 50 to 100% polymorphism in banding pattern, indicating its effectiveness in evaluating the genetic diversity in *P. glaucum*. The primers OPA-02 and OPP-09 were the most informative primer with the maximum PIC values of 90.8 and 89%, respectively, and can serve as robust genetic markers in genetic diversity analysis of *P. glaucum*.

The RAPD profiles generated by these two informative primers are shown in Figs 2a and b. The combined dendrogram of 12 random primer data matrices delineated 20 MS lines into four main clusters and one as out group (Fig. 3). Cluster I had six MS lines, of which CZMS-1A, 17A and 19A formed a sub-cluster

together and three MS lines, viz., CZMS-5A, 7A and 15A, formed another sub-cluster. Cluster II comprised of six MS lines, viz., CZMS-3A, 12A, 4A, 20A, 9A and 13A. Cluster III included four MS lines, viz., CZMS-8A, 18A, 11A and 14A. Cluster IV grouped CZMS-2A, 6A and 21A together, whereas CZMS-22A formed the most distinct out group to these clusters. Earlier researchers have also reported usefulness of RAPD as robust molecular markers in assessing genetic diversity for crop improvement in pearl millet^{13,26}.

All the 20 novel gene sequences exhibiting nucleotide variations have been submitted to NCBI database and assigned accession numbers KM288868

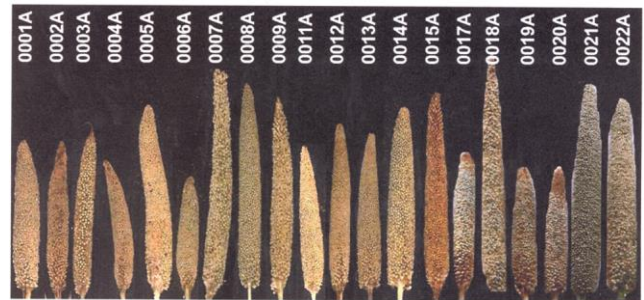


Fig. 1 — A pictorial view of ear head diversity in MS lines of *P. glaucum*.

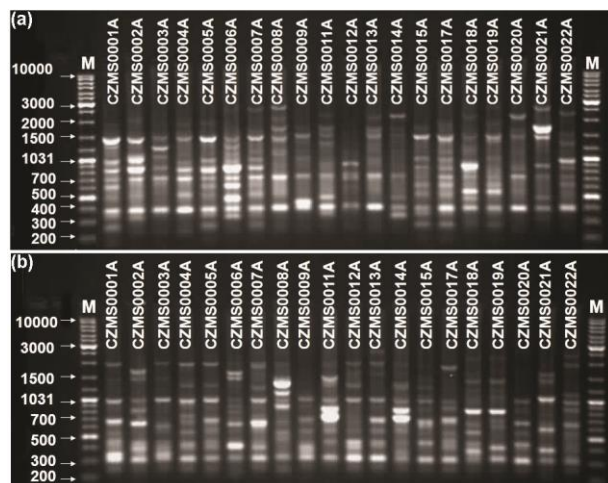
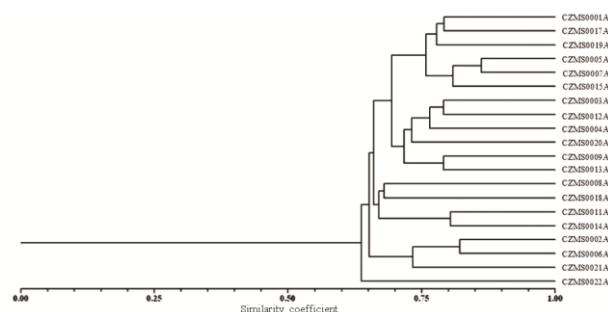
Table 2 — Morphological characterization of MS lines of *P. glaucum* developed at CAZRI, Jodhpur

No.	MS lines	DF	PHT	EL	EG	ETill	EHD No.	EHDWT	GY	500 GWT	PHI
1	CZMS0001A	48	110	14	2.0	2	62	743	374	3.6	0.50
2	CZMS0002A	51	83	14	1.7	2	65	649	412	3.0	0.64
3	CZMS0003A	48	85	15	1.4	3	95	868	523	4.1	0.60
4	CZMS0004A	53	119	12	1.6	2	73	616	265	3.7	0.42
5	CZMS0005A	51	151	17	1.7	2	61	1002	592	3.4	0.59
6	CZMS0006A	51	99	11	1.8	2	65	654	376	3.3	0.57
7	CZMS0007A	47	129	19	1.5	3	89	1042	597	4.1	0.57
8	CZMS0008A	46	174	17	1.7	3	104	1666	967	3.4	0.58
9	CZMS0009A	40	124	16	1.2	6	138	1078	734	4.3	0.68
10	CZMS0011A	46	116	13	1.6	2	58	805	562	3.7	0.69
11	CZMS0012A	48	120	15	1.5	2	51	522	197	3.3	0.39
12	CZMS0013A	53	138	14	1.5	3	81	1093	717	3.5	0.66
13	CZMS0014A	49	148	16	1.9	3	101	1871	1222	3.8	0.65
14	CZMS0015A	45	126	17	1.5	3	66	557	365	3.7	0.65
15	CZMS0017A	48	81	13	1.5	2	79	900	463	4.0	0.51
16	CZMS0018A	49	83	20	1.5	2	62	651	315	3.9	0.48
17	CZMS0019A	49	88	12	1.5	2	88	915	479	4.1	0.53
18	CZMS0020A	48	92	12	1.4	2	73	837	494	3.9	0.60
19	CZMS0021A	44	179	18	1.9	3	112	2481	1855	4.0	0.75
20	CZMS0022A	53	164	16	1.8	3	73	977	591	3.1	0.61
	CD 5%	2	18	2	0.3	1	25	218	153	0.7	0.09

DF: Days to flower, PHT: Plant height, EL: Ear head length, EG: Ear head girth, ETill: Effective tillers, EHDNo.: Effective head number, EHDWt: Ear head wt, GY: Grain yield, 500 GWT: 500 grain wt, PHI: Panicle harvest index

Table 3 — Details of RAPD primers, polymorphism and PIC values among MS lines of *P. glaucum*

No.	Primer code	Primer sequence	GC (%)	Mol wt (bp)		No. of bands	No. of polymorphic bands	Polymorphism (%)	PIC value (%)
				Min.	Max.				
1.	OPA-02	TGC CGA GCT G	70	275	2500	14	13	92.6	90.8
2.	OPA-03	AGT CAG CCA C	60	350	1600	9	9	100.0	86.4
3.	OPA-06	GGT CCC TGA C	70	325	2050	12	8	66.7	87.2
4.	OPA-09	GGG TAA CGC C	70	300	1900	12	11	91.7	89.0
5.	OPA-13	CAG CAC CCA C	70	500	1400	5	3	60.0	66.2
6.	OPA-16	AGC CAG CGA A	60	400	2000	13	12	92.3	86.2
7.	OPB-05	TGC GCC CTT C	70	400	3000	11	10	90.9	83.1
8.	OPB-06	TGC TCT GCC C	70	450	2000	10	7	70.0	86.1
9.	OPB-10	CTG CTG GGA C	70	350	2000	6	3	50.0	76.6
10.	OPB-14	TCC GCT CTG G	70	425	2000	11	11	100.0	83.5
11.	OPP-06	GTG GGC TGA C	70	350	2400	9	7	77.8	76.4
12.	OPP-14	CCA GCC GAA C	70	400	2000	9	9	100.0	80.4

Fig. 2 (a & b) — RAPD profiles of MS lines of *P. glaucum* amplified by (a) OPA-02, & (b) OPA-09 primers.Fig. 3 — Dendrogram of MS lines of *P. glaucum* based on RAPD informative primers.

to KM288887. The nucleotide base pair length of nuclear ribosomal RNA gene of 20 MS lines of *P. glaucum* are shown in Table 4. The conserved 5.8S rDNA region was recorded with uniform nucleotide length of 160 bp in all the MS lines; while ITS-2

Table 4 — Nucleotide base pair lengths of nuclear rRNA gene of MS lines of *P. glaucum*

Genotype	Gen acc. no.	ITS-1 (bp)	5.8S (bp)	ITS-2 (bp)	Total (bp)
CZMS0001A	KM288868	208	160	219	587
CZMS0002A	KM288869	207	160	219	586
CZMS0003A	KM288870	207	160	219	586
CZMS0004A	KM288871	207	160	219	586
CZMS0005A	KM288872	207	160	219	586
CZMS0006A	KM288873	207	160	219	586
CZMS0007A	KM288874	207	160	219	586
CZMS0008A	KM288875	206	160	219	585
CZMS0009A	KM288876	207	160	219	586
CZMS0011A	KM288877	207	160	219	586
CZMS0012A	KM288878	207	160	219	586
CZMS0013A	KM288879	207	160	219	586
CZMS0014A	KM288880	207	160	219	586
CZMS0015A	KM288881	206	160	219	585
CZMS0017A	KM288882	207	160	219	586
CZMS0018A	KM288883	207	160	219	586
CZMS0019A	KM288884	207	160	219	586
CZMS0020A	KM288885	207	160	219	586
CZMS0021A	KM288886	207	160	219	586
CZMS0022A	KM288887	207	160	219	586

region was recorded with 219 bp length in all the MS lines. Substantial variation was recorded in ITS-1 region ranging 206 to 208 bp. Accordingly the total length of complete 5.8S gene region also varied 585 to 587 bp. Upon multiple sequence alignment of nucleotide sequences of all the 20 MS lines together we detected SNPs at 16 places in ITS-1 and 2 places in ITS-2 region. In addition to this, INDEL (small insertion and deletion) was also detected at 44th position in ITS-1 region, whereas no SNP or INDEL

were recorded in highly conserved 5.8S rRNA gene region. Under the present study, a high degree of nucleotide sequence diversity by way of SNPs, INDELs and ITS length polymorphism allowed separation of all the 20 MS lines of *P. glaucum*. Substantial intra-specific genetic diversity was validated by significant bootstrap values indicating phylogenetic utility of ITS-1 region, particularly, in developing bar code of pearl millet germplasm. The boot strap values are dependable measures of phylogenetic accuracy and higher values are likely to indicate reliable grouping²⁷.

The multiple sequence alignment of 20 MS lines of *P. glaucum* generated a phylogram using clustal X 2.0.22 and NJ plot (Fig. 4). The perusal of phylogram further delineated *P. glaucum* MS lines into three main clusters with CZMS-21A as distinct out group. Cluster I included two MS lines (CZMS-8A, CZMS-15A), cluster II three MS lines (CZMS-12A, 2A, 6A) and Cluster III rest of 14 MS lines with significant delineation from each other. Most of the pearl millet hybrids developed are based upon A₁-based CMS as a source, whereas CZMS-21A was developed using A₄-based CMS system and, therefore, expected to be more stable. Among the different CMS systems, A₄ and A₅ were also reported to be the most stable²⁸.

The perusal of morpho-physiological markers *vis-à-vis* genetic markers, *i.e.*, RAPD and ITS region analysis, exhibited no consistency in grouping 20 MS lines of *P. glaucum*. With similar morphological traits as phenotypically similar varieties were genetically catalogued into different clusters of RAPD dendrogram and ITS phylogram. It is evident from the fact that CZMS-21 and CZMS-14 shared similar morpho-physiological characters with significantly higher ear head girth, number of ear heads/plot and

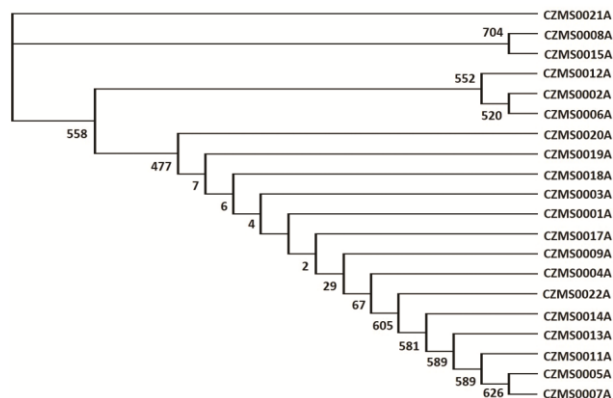


Fig. 4 — Phylogram generated using NJ plot of multiple sequence alignment of rDNA region of MS lines of *P. glaucum*.

grain wt but were delineated into different phylogenetic clusters of RAPD and that of phylogram.

Five *P. glaucum* MS populations were subjected to PCA. The first three principal coordinates accounted for 26.13, 22.01 and 14.12%, respectively amounting to a total of 62.26% of total variance, indicating the presence of wide genetic diversity among *P. glaucum* MS populations studied. The Eigen vector analysis indicated that the contributions of the first three factors were 11.68, 9.84 and 6.31, respectively, explaining a total of 27.83% of total variability. A perusal of the distribution of five populations across principal coordinates clearly exhibited that population one is widely distributed across all the three principal coordinates as compared to others (Fig. 5). Analysis of molecular variance of RAPD data (Table 5) revealed that a greater proportion of total genetic variation existed within population (87%) rather than among population (13%). The results validate the existence of higher genetic diversity among *P. glaucum* MS lines.

The population of pedigree one (CZBC CO-60) was widely distributed across all the three principal coordinates as compared to others. The least genetic distances between the MS line pedigree population 1 (CZBC CO-60) and population 3 (ICMB 95111 × [843B × EEBC-S1-407]) could be attributed to similar genetic background, whereas the maximum genetic distances between pedigree population 4 (ICMB 94555 × ICMB 96333) and population 5 (CZBC CO-19) were due to the largest variable genetic background.

Table 5 — Summary AMOVA of five pedigree populations of *P. glaucum*

Source	df	SS	MS	Est. var.	%
Among pops	4	93.076	23.269	2.300	13
Within pops	16	237.400	14.838	14.838	87
Total	20	330.476		17.137	100

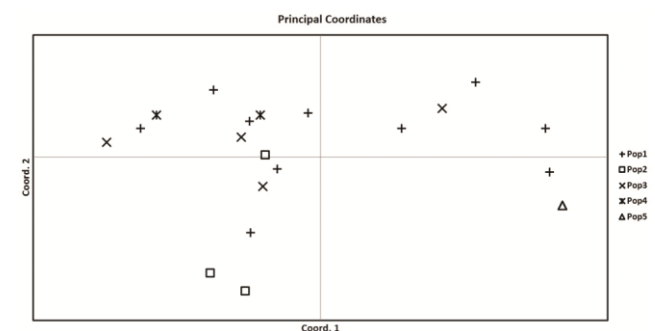


Fig. 5 — The principal coordinates analysis (PCA) of populations of *P. glaucum* using GenAlEx software.

Table 6—Summary of genetic variation statistics for all loci of five pedigree populations of *P. glaucum*

Locus (mean)	Sample size	na	ne	h	i
Pop 1	9	1.6446	1.3273	0.1987	0.3048
Pop 2	3	1.3967	1.2826	0.1594	0.2330
Pop 3	4	1.4793	1.2311	0.1470	0.2295
Pop 4	2	1.2893	1.2045	0.1198	0.1749
Pop 5	1	1.0000	1.0000	0.0000	0.0000
Mean of all loci	19	0.2337	0.1250	0.4651	0.5750

na: Observed number of alleles, ne: Effective number of alleles, h: Nei's gene diversity, i: Shannon information index

Table 7—Matrix of unbiased genetic identity and genetic distance according to Nei (1973)²⁹ among five populations of *P. glaucum* based on RAPD markers

Pop	1	2	3	4	5
1	****	0.9346	0.9731	0.9339	0.7990
2	0.0676	****	0.9396	0.8951	0.7670
3	0.0272	0.0623	****	0.9214	0.7978
4	0.0684	0.1108	0.0818	****	0.7516
5	0.2244	0.2653	0.2259	0.2855	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

A summary of mean genetic variation statistics of all the five populations along with the mean of all loci is presented in Table 6. The mean value of all the five populations together for Nei's gene diversity (*h*) was 0.461 and Shannon information index (*i*) was 0.575. Results showed that the genetic diversity of *P. glaucum* MS lines of population one was the richest among all the five populations. Nei's unbiased measure of genetic distance was employed to further elucidate the gene differentiation among populations (Table 7). The Nei genetic distance ranged 0.0272 to 0.2855 and the genetic identity ranged 0.7516 to 0.9731. The largest genetic distance was measured between populations 4 and 5 and the least between populations 1 and 3.

Genetic structure estimates for the five populations of *P. glaucum* representing different pedigrees showed that the total genetic diversity (H_T) was 0.2337, genetic diversity within each population (H_S) was 0.1250, coefficient of gene differentiation (G_{ST}) was 0.4651 and the level of gene flow (N_m) was 0.5750. Higher G_{ST} (0.4651) reflects the presence of higher amount of differentiation over multiple loci among MS populations. The level of N_m (0.5750) demonstrates a very high migration rate of alleles or genes from one

population to another MS population. Migration of genes/alleles into or out of a population may be responsible for a marked change in allele frequencies, which may result in the addition of new genetic variants to the established gene pool of *P. glaucum* population.

References

1. FAO, *Bulletin of statistic*, vol 1 (Statistics Division, Food and Agriculture Organization, Rome) 2000, 16-36.
2. Reddy A A, Yadav O P, Malik D P, Singh I P, Ardeshta N J *et al*, Utilization pattern, demand and supply of pearl millet grain and fodder in western India, *Working Paper Ser No. 37* (International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India) 2013, 24.
3. Kelley T G, Rao P P, Weltzien R E & Purohit M L, Adoption of improved cultivars of pearl millet in an arid environment: Straw yield and quality considerations in western Rajasthan, *Expt Agric*, 32 (1996) 161-171.
4. Yadav O P & Weltzien-Rattunde E, New pearl millet populations for Rajasthan, India, *Integr Systems Proj Rep No. 10* (International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India) 1998, 88.
5. Virk D S, Biometrical analysis in pearl millet—A review, *Crop Improv*, 15 (1988) 1-29.
6. Bidinger F R & Parthasarathy-Rao P, Genetic and cultural improvement in the production of pearl millet, in *Proc Intl Congr Plant Physiol*, edited by S K Sinha, P V Sane, S C Bhargava & R K Agarwal (Society for Plant Physiology and Biochemistry, New Delhi, India) 1990, 194-206.
7. Manga V K, Ability of new pearl millet restorers to produce high yielding hybrids for moisture stress environments, *Ann Arid Zone*, 47 (2008) 107-110.
8. Manga V K, Components of genetic variance and interrelationship among quantitative traits in CAZRI bred inbred restorers of pearl millet, *Electronic J Plant Breed*, 4 (2013) 1325-1330.
9. Manga V K & Kumar A, Developing pearl millet seed parents adapted to arid regions of north-western India, *Ann Arid Zone*, 52 (2013) 71-75.
10. Williams J G K, Kubelik A R, Livak K J, Rafalski J A & Tingey S V, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, *Nucleic Acids Res*, 18 (1990) 6231-6235.
11. Raturi A, Singh S K, Sharma V & Pathak R, Molecular characterization of *Vigna radiata* (L.) Wilczek genotypes based on nuclear ribosomal DNA and RAPD polymorphism, *Mol Biol Rep*, 39 (2012) 2455-2465.
12. Pathak R, Singh S K, Singh M & Henry A, Molecular assessment of genetic diversity in clusterbean (*Cyamopsis tetragonoloba*) genotypes, *J Genet*, 89 (2010) 243-246.
13. Shah A K, Ravikiran R, Litoriya N S, Patel H K & Talati J G, Validation of genetic purity among hybrid lines in pearl millet (*Pennisetum glaucum* L.) using RAPD and SSR markers, *Indian J Agric Biochem*, 26 (2013) 172-176.
14. Alvarez I & Wendel J F, Ribosomal ITS sequences and plant phylogenetic inference, *Mol Phylogenet Evol*, 29 (2003) 435-455.
15. Singh S K, Tiwari M, Kamal S & Yadav M C, Morel phylogeny and diagnostics based on restriction fragment length polymorphism analysis of ITS region of 5.8S ribosomal DNA, *J Biochem Biotechnol*, 14 (2005) 179-183.

- 16 Kakani R K, Singh S K, Pancholy A, Meena R S, Pathak R *et al*, Assessment of genetic diversity in *Trigonella foenum-graecum* based on nuclear ribosomal DNA, internal transcribed spacer and RAPD analysis, *Plant Mol Biol Rep*, 29 (2011) 315-323.
- 17 Saini A, Reddy S K & Jawali N, Intra individual and intra species heterogeneity in nuclear rDNA ITS region of *Vigna* species from subgenus *Ceratotropis*, *Genet Res*, 90 (2008) 299-316.
- 18 Mir B A, Koul S, Kumar A, Koul M K, Soodan A S *et al*, Intraspecific variation in the internal transcribed spacer (ITS) regions of rDNA in *Withania somnifera* (Linn.) Dunal, *Indian J Biotechnol*, 9 (2010) 325-328.
- 19 Carvalho A, Guedes-Pinto H & Lima-Brito J, Genetic diversity among old Portuguese bread wheat cultivars and botanical varieties evaluated by ITS rDNA PCR-RFLP markers, *J Genet*, 88 (2009) 363-367.
- 20 White T J, Bruns T, Lee S & Taylor J, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in *PCR protocols: A guide to methods and applications*, edited by M A Innis, D H Gelfand, J J Sninsky & T J White (Academic Press, Inc., San Diego, CA, USA) 1990, 315-322.
- 21 Peakall R & Smouse P E, GenALEX 6: Genetic analysis in excel: Population software for teaching and research, *Mol Ecol Notes*, 6 (2006) 288-295.
- 22 Nei M, Definition and estimation of fixation indices, *Evolution*, 40 (1986) 643-645.
- 23 Slatkin M & Barton N H, A comparison of three indirect methods for estimating average levels of gene flow, *Evolution*, 43 (1989) 1349-1368.
- 24 Bikash A, Yadav I S & Arya R K, Studies on variability, correlation and path analysis in pearl millet, *Forage Res*, 39 (2013) 134-139.
- 25 Yadav O P, Weltzien-Rattunde E, Bidinger F R & Mahalakshmi V, Heterosis in landrace-based top cross hybrids of pearl millet across arid environments, *Euphytica*, 112 (2000) 285-295.
- 26 Govindaraj M, Selvi B, ArunPrabhu D & Rajarathinam S, Genetic diversity analysis of pearl millet (*Pennisetum glaucum* [L.] R.Br.) accessions using molecular markers, *Afr J Biotechnol*, 8 (2009) 6046-6052.
- 27 Hillis D M & Bull J J, An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analyses, *Syst Biol*, 42 (1993) 182-192.
- 28 Rai K N, Khairwal I S, Dangaria C J, Singh A K & Rao A S, Seed parent breeding efficiency of three diverse cytoplasmic-nuclear male sterility systems in pearl millet, *Euphytica*, 165 (2009) 495-507.
- 29 Nei M, Analysis of gene diversity in subdivided populations, *Proc Natl Acad Sci USA*, 70 (1973) 3321-3323.