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Assessment of Molecular Diversity Among Pearl Millet [*Pennisetum glaucum* (L.) R. Br.] Maintainer (B) and Restorer Lines

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Abstract: Pearl millet is predominantly cultivated in western arid and semi-arid regions of India. Pearl millet productivity has drastically increased in the last seven decades mainly due to adoption of hybrids. Hybrid development program mainly depends upon exploitation of diverse genetic stock. In the present study a set of 48 maintainer lines (B) and 52 restorer lines were assessed for molecular diversity using PCR based RAPD and ISSR markers. The Jaccard's similarity coefficient was used to create pair-wise comparisons of distinct and shared polymorphic amplification products. The dendrogram represented the classification of all 100-pearl millet genotypes into two major groups. First group was formed with single genotype J-2296 and second group consisted of 99 genotypes further grouped in three sub-clusters. In second major cluster, maximum genotypes were grouped in subcluster I (45) followed by subcluster II (35) and subcluster III (19). Grouping of B lines and R lines were random and no specific grouping was observed. The observed divergence can be useful for selecting B lines and R lines from different clusters for developing hybrids. J-2296 seems to be highly diverse from rest of the genotypes, it can be used for hybrid development as well as for R line improvement.

Key words: Pearl millet, RAPD, ISSR, dendrogram.

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Pearl millet [*Pennisetum glaucum* (L.) R. Br.], is the sixth important cereal crop after rice, wheat, maize, barley and sorghum. It is cultivated in 30 mha in more than 30 countries and support livelihood of more than 100 million people (Yadav *et al*., 2012). Major pearl-millet producing countries are India, Senegal, Mali, Burkina Faso, Niger, Nigeria, Chad and Sudan. India is the largest producer of this crop with the national productivity being 1.2 tons ha^{-1} (Yadav *et al.,* 2021). Major pearl millet growing states in India are Rajasthan, Maharashtra, Gujarat, Uttar Pradesh, Haryana, Karnataka, Madhya Pradesh, Tamil Nadu and Andhra Pradesh. Pearl millet generally occupies the drier parts of the semi-arid tropics and extends into the arid zone and it is well adapted to low soil fertility

and high temperature situations. Pearl millet is a C4 species with a very high photosynthetic efficiency and dry-matter production. It is an important source of fodder and source of feed in dry months (Hash *et al*., 2003). With regard to nutritional quality, it is equivalent to maize and superior to sorghum in protein content, quality, protein efficiency ratio, metabolizable energy levels and does not contain tannins. Pearl millet contains more than 35% of lysine than sorghum (Rooney and McDonough, 1987). It contains 5-6% oil (Jambunathan and Subramanian, 1988) and is rich source of iron and zinc (Manwaring *et al.,* 2016). Besides, it is also used as main commodity in poultry feed and its use is increasing gradually. Due to presence of many nutrients and higher proportion of insoluble dietary fiber, which causes slow release of sugar, making the food products especially for diabetic patients. It is suitable for large-scale manufacture of various food products such as traditional meals, baby foods, snack foods and dietary foods in both grain and flour form (Rai *et al.,* 2008; Sharma and Choudhary, 2012; Jukanti *et al.,* 2016). It is having high nourishment qualities, the bioavailability of various minerals such as calcium, phosphorous and trace elements such as zinc, iron, copper and manganese are high in pearl millet (Satyavathi *et al.,* 2022).

Breeding strategies has gone long way in pearl millet, the genetic gains observed in pearl millet is more than 300%; i.e., in 1950-54 it was 303 kg ha $^{-1}$ which gradually reached 1239 kg ha^{-1} during 2015-2019 (Yadav *et al.,* 2021). Development of hybrids contributed significantly in enhancing the productivity status in the country. Still the productivity levels are low in the hot arid regions of the country as the environment is highly harsh and water being the limiting factors leading to yield penalty. Breeding for hot arid and semi arid region is major objective for pearl millet improvement. Selection of diverse parents rewards by exploiting heterosis in the crop; diversity assessment based on morphological traits is heavily influenced by the environment, thus use of molecular tools gives a genomic view of the diversity resting in the gene pool. A notable milestone in applications of molecular markers in pearl millet was the construction of the first genetic map in 1993 using RFLP markers (Liu *et al.,* 1994); though RFLP and AFLP are robust, they involve Arduous methods and usage of radioactive materials. The polymerase chain reaction (PCR)-based, quick, and radioactive-chemical-free RAPD and ISSR are preferred. There are no blotting or hybridizing phases in the RAPD and ISSR method. RAPD and ISSR markers provide a number of benefits, including a higher frequency of polymorphism, quickness, technical simplicity, the need for only a few nanograms (ng) of DNA, the absence of the need for prior knowledge of the DNA sequence, and the viability of automation (Fahima *et al.,* 1999). When it comes to revealing genetic diversity at the genomic level, RAPD and ISSR are highly effective (Jaya Prakash *et al.,* 2006; Tomar *et al.,* 2014a; Tomar *et al.,* 2014b). Molecular markers can be used in pearl millet breeding for multiple environmental stresses (Satyavathi *et al.,* 2019). So, the goal of the current study was to examine genotypes in order to determine the degree of molecular variation among the pearl millet maintainer (B) line and restorer (R) lines for generating information on diversity for selecting parents for hybrid breeding.

Material and Methods

Plant material: A set of hundred genotypes comprising 48 maintainer (B) lines and 52 restorer (R) lines were collected from Main Pearl Millet Research Station, Jamnagar, Gujarat (Table 1). The genotypes were sown in the plastic cup for germination. The young leaves were taken from all the hundred genotypes and were frozen in the liquid nitrogen and stored at -80°C for further use.

DNA Isolation and Quantification: The Doyle and Doyle (1990) method was improved to isolate whole genomic DNA using cetyl trimethyl ammonium bromide (CTAB) with a few minor adjustments to the amount of leaf tissue used, the NaCl content, the length of the incubation, and the temperature. With a mortar and pestle that had already been cold, 0.5-1 g of leaves were pounded into a fine powder using liquid nitrogen. In a 2 ml micro centrifuge tube, ground leaf tissues were transferred together with extraction buffer (1.4 M NaCl, 100 mM Tris HCl (pH-8), 20 mM EDTA, 2% CTAB, and 0.5% PVP). For one hour, this mixture was incubated at 65°C. The tubes were centrifuged for 5 minutes at 4°C at 8000 rpm, and the supernatant was then transferred

Table 1. List of pearl millet genotypes studied (Sr. no: 1-48 B Lines; 49-100 R Lines)

1.	JMSB101	26.	221-BCS-19	51.	J-2296	76.	J-2584
2.	JMSB20101	27.	223-BCS-19	52.	J-2340	77.	J-2585
3.	JMSB20102	28.	227-BCS-19	53.	J-2372	78.	J-2556
4.	JMSB20111	29.	229-BCS-19	54.	J-2405	79.	J-2587
5.	JMSB20143	30.	231-BCS-19	55.	J-2454	80.	J-2588
6.	JMSB20151	31.	233-BCS-19	56.	J-2467	81.	J-2589
7.	JMSB20152	32.	235-BCS-19	57.	J-2480	82.	J-2590
8.	JMSB20154	33.	237-BCS-19	58.	J-2500	83.	J-2591
9.	JMSB20155	34.	239-BCS-19	59.	J-2517	84.	J-2592
10.	JMSB20156	35.	243-BCS-19	60.	J-2526	85.	J-2593
11.	JMSB20158	36.	245-BCS-19	61.	J-2532	86.	J-2594
12.	JMSB20159	37.	247-BCS-19	62.	J-2539	87.	J-2595
13.	JMSB20161	38.	249-BCS-19	63.	J-2552	88.	J-2596
14.	JMSB20171	39.	251-BCS-19	64.	J-2553	89.	J-2597
15.	JMSB20172	40.	253-BCS-19	65.	J-2562	90.	J-2598
16.	JMSB20173	41.	255-BCS-19	66.	J-2563	91.	J-2599
17.	JMSB20174	42.	257-BCS-19	67.	J-2565	92.	J-2601
18.	JMSB20175	43.	259-BCS-19	68.	J-2566	93.	J-2602
19.	JMSB20181	44.	261-BCS-19	69.	J-2569	94.	J-2603
20.	JMSB20191	45.	263-BCS-19	70.	J-2571	95.	J-2604
21.	JMSB20192	46.	265-BCS-19	71.	J-2576	96.	J-2606
22.	JMSB20193	47.	267-BCS-19	72.	J-2579	97.	J-2607
23.	215BCS-19	48.	271-BCS-19	73.	J-2580	98.	J-2611
24.	217-BCS-19	49.	J-104	74.	J-2582	99.	J-2619
25.	219-BCS-19	50.	J-2290	75.	J-2583	100.	J-2627

to a clean 2 mL micro centrifuge tube. An equal volume of chilled chloroform: isoamyl alcohol (24:1) was added, mixed by gentle inversion and centrifuged at 10,000 rpm for 10 min at 4°C. The upper aqueous phase was transferred to a fresh micro centrifuge tube, and DNA was precipitated by adding an equal volume of pre-chilled iso-propanol. This procedure was then centrifuged at 12,000 rpm for 15 minutes at 4°C after the upper aqueous phase was incubated at -20°C for an hour. The supernatant was discarded, and 70% ethanol was used to wash the particle. After being air dried, the pellet was re-dissolved in 100 l of Tris-EDTA (TE) buffer. Each sample was diluted with TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0) to 50 ng l^{-1} before being placed in storage at 4°C.

The quantity and quality of DNA was analyzed by spectrophotometrically method at 260 nm and 280 nm. Purity of DNA was also checked by using 0.8% agarose gel, stained with ethidium bromide, visualized under a UV transilluminator and photographed using the SYNGENE G:BOX. Concentration of DNA can be calculated by following formula: DNA concentration (µg ml^{-1}) = OD at 260 nm x dilution factor x 50 µg ml^{-1}.

RAPD amplification: 30 primers (decamer oligonucleotides) were employed in total to test the fidelity of the amplification; those that performed the best in terms of the quantity of bands produced per reaction were chosen for further examination. Amplification of RAPD fragments made from random decamer primers in a 25 µl total reaction volume that also contains 50 ng of genomic DNA, 0.6 l of 10 mM dNTPs, 1 U of Taq DNA polymerase from Invitrogen, and 2 µl of 10 pmol primer.

The following temperature profile was used to conduct PCR reactions in a Viriti thermo cycler (Life Technology): a 5-minute initial denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 45 sec at 44° to 52°C (varying depending on the primer Tm value), 2 min at 72°C, and a final 10-minute extension at 72°C.

Each sample's entire reaction mixture was placed onto 1.2% agarose gels and run in TBE buffer at 120 V for 100 minutes. The gels were stained with 0.5 g ml^{-1} ethidium bromide. Reference was made using a 100-bp DNA ladder that produced 15 bands ranging in size from 100 to 1500 bp. Using a G:box Chemi XR 5 (Syngene) gel document, PCR products were seen.

ISSR amplification: ISSR amplification reactions were carried out in 20 µl volume containing 1µl DNA (40 ng), 10× reaction buffer (Invitrogen; 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl), 0.6 µl of 10mM dNTPs and 1 unit of Taq DNA polymerase (Invitrogen). Twenty different UBC series primers (The University of British Columbia, Vancouver, Canada) were tested and those that gave the best performance in terms of number of bands per reaction were selected. The amplification reaction consisted of an initial denaturation step at 94°C for 5min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at a specific annealing temperatures and 2 min at 72°C (extension) followed by a final extension step at 72°C for 5 min. Amplification products were electrophoresed in 1.5% agarose in 1×TBE buffer.

The gels were documented using the gel documentation system and stained with ethidium bromide. With each primer, each experiment was run twice, and the primers that produced reproducible fingerprints were taken into consideration for data analysis.

Molecular data Analysis: The RAPD and ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. By comparing the banding patterns of genotypes for a specific primer, genotype-specific bands were identified. Faint or unclear bands were not considered. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Pairwise similarity matrices were generated by Jaccard's coefficient of similarity by using the SIMQUAL format of NTSYSpc (Rohlf, 1993). A dendrogram was constructed by using the unweighted pair group method with arithmetic average (UPGMA) with the SAHN module of NTSYSpc2.02e to show genetic relationships as revealed by the similarity coefficient.

Results and Discussion

The set of 100 pearl millet genotypes were assessed for molecular diversity using PCR based molecular markers; these markers are random and dominant in nature. Based on molecular diversity prediction of heterosis can be made (Passoupathy *et al.*, 2015). Assessing diversity between B and R lines can generate valuable information for selecting diverse parents for heterosis breeding.

Thirty RAPD primers (Table 2; serial no. 1 to 30) were selected for genomic comparison; amplification of genomic DNA yielded 221 fragments that could be scored. All the primers used in this study, gave amplification with 7.36 average number of bands per primer. The number of fragments ranged from 4 (OPA-5) to 11 (OPA-7) with band size varying between 180 to 1700 bp. From the 221 amplified bands, 162 were polymorphic, with 5.4 average number of polymorphic bands per primer. Percentage polymorphism ranged from 33.3% (OPD-5 and OPI-2) to a maximum of 100% (10 primers), with an average of 72.96% polymorphism. Out of total 30 primers, 15 primers showed per cent polymorphism less than 75% with average resolving power of 8.89. The resolving power (Rp) of the thirty RAPDs primers ranged from 4.27 (OPE-5) to 13.69 (OPG-2).

Twenty ISSR primers (Table 2; serial no. 31 to 50) amplified 1194 total number of amplified bands. The total number of polymorphic bands were 119 and total number of monomorphic bands were 22 making the tally of 134 total number of bands. All the primers used in this study, gave amplification with 6.7 average number of bands per primer and 5.95 average number of polymorphic bands per primer. The number of fragments ranged from 2 (ISSR15) to 13 (ISSR4) with band size varying between 120 to 1550 bp. Percentage polymorphism ranged from 33.3% (ISSR7 and ISSR8) to a maximum of 100% (ISSR1, ISSR4, ISSR5, ISSR9, ISSR11, ISSR14, ISSR15, ISSR16, ISSR17, ISSR18, ISSR19, and ISSR20), with an average of 85.02% polymorphism. The resolving power (Rp) of the twenty ISSR primers ranged from 1.69 (ISSR15) to 13.39 (ISSR4).

Table 2. RAPD and ISSR primers details and details of band profiles generated in pearl millet genotypes

Sr. No	Primer series	Sequence 5′-3′	Tm (°C)	Total number of band	Number of polymorphic bands	Number of monomorphic bands	Percentage of polymorphism	Total Number of bands amplified	Resolution power
1	OPA-1	CAGGCCCTTC	38.2	9	9	0	100	62	9.53
2	OPA-2	TGCCGAGCTG	42.4	12	12	0	100	193	10.72
3	OPA-3	AGTCAGCCAC	26.8	8	3	5	37.5	63	9.092
4	OPA-5	AGGGGTCTTG	31.8	4	4	0	100	111	6.17
5	OPA-7	GAAACGGGTG	34.5	11	5	6	45.4	35	13.84
6	OPA-8	GTGACGTAGG	22.9	10	7	3	70	61	10.30
7	OPA-10	GTGATCGCAG	29.8	10	8	2	80	78	11.691
8	OPB-5	TGCGCCCTTC	45.6	7	6	1	85.7	78	11.47
9	OPB-6	TGCTCTGCCC	40.2	7	4	3	57.1	22	6.471
10	OPB-8	GTCCACACGG	34.3	7	7	0	100	56	8.61
11	OPD-5	TGAGCGGACA	36.9	6	2	4	33.3	37	9.01
12	OPD-1	ACCGCGAAGG	44.1	6	4	2	66.6	43	6.27
13	OPD-3	GTCGCCGTCA	41.4	5	5	0	100	104	5.78
14	OPE-2	GGTGCGGGAA	44.9	7	4	3	57.1	49	7.1
15	OPE-3	CCAGATGCAC	29.2	5	2	3	40	41	6.30
16	OPE-5	TCAGGGAGGT	31.5	5	4	1	80	38	4.27
17	OPF-4	GGTGATCAGG	25.0	8	6	2	75	232	12.89
18	OPF-7	CCGATATCCC	25.0	6	4	2	66.6	70	10.77
19	OPG-2	GGCACTGAGG	27.0	8	3	5	37.5	89	13.69
20	OPG-5	CTGAGACGGA	25.0	8	8	0	100	49	7.53
21	OPH-4	GGAAGTCGCC	27.0	6	6	0	100	49	7.53
22	OPI-2	GGTGGAGAGG	27.0	6	2	4	33.3	35	8.95
23	OPJ-2	CCCGTTGGGA	27	7	7	0	100	39	6.01
24	OPK-2	GTCTCCGCAA	25	7	7	0	100	58	8.92
25	OPK-5	TCTGTCGAGG	25	9	9	0	100	70	10.22
26	OPK-6	CACCTTTCCC	25	8	6	2	75	73	11.23
27	OPL-8	AGCAGGTGGA	25	6	4	2	66.6	66	10.153
28	OPL-12	GGGCGGTACT	25	7	4	3	57.14	34	5.23
29	OPM-2	ACAACGCCTC	25	8	5	3	62.5	45	6.9
30	OPM-3	GGGGGATGAG	27	8	5	3	62.5	66	10.15
31	ISSR1	GTGTGTGTGTGTGTAT	42	9	9	0	100	275	15.28
32	ISSR2	GGAGAGGAGAGGAGA	49	4	8	3	72.7	44	8.08
33	ISSR3	GAGAGAGAGAGAGAGAGAAT	50	5	4	1	80	130	7.22
34	ISSR4	CACACACACACACACAA	52	13	13	0	100	241	13.39
35	ISSR5	ACACACACACACACACG	54	5	5	0	100	107	5.94
36	ISSR6	AGAGAGAGAGAGAGAGT	42	9	4	5	44.4	35	11.13
37	ISSR7	GAGAGAGAGAGAGAGAT	42	6	2	4	33.3	35	8.95
38	ISSR8	ATGATGATGATGATGATG	51	6	2	4	33.3	42	8.82
39	ISSR9	CACACACACACACACAA	52	7	7	0	100	164	9.11
40	ISSR10	CACACACACACACACAG	52	7	6	1	85.7	159	8.83
41	ISSR11	AGAGTTGGTAGCTCTTGA	55	5	5	0	100	104	5.78
42	ISSR 12	ATATATATATATATATYC	21	7	5	2	71	72	7.31

Table 2. Contd...

Sr. No.	Primer series	Sequence 5′-3′	Tm (°C)	Total number of band	Number of poly-morphic bands	Number of mono-morphic bands	Percentage of poly-morphism	Total Number of bands ampli-fied	Resolu-tion power
43	ISSR13	ATATATATATATATATYG	25	10	8	2	80	38	4.77
44	ISSR14	AGAGAGAGAGAGAGAGYT	49	7	7	0	100	16	2.46
45	ISSR15	AGAGAGAGAGAGAGAGYC	42.7	2	2	0	100	11	1.69
46	ISSR16	AGAGAGAGAGAGAGAGYA	40.8	9	9	0	100	62	9.53
47	ISSR17	TATATATATATATATART	25.8	5	5	0	100	136	7.56
48	ISSR18	TATATATATATATATARC	19.5	7	7	0	100	156	8.67
49	ISSR19	TATATATATATATATARG	22.9	7	7	0	100	56	8.61
50	ISSR20	GAGAGAGAGAGAGAGAYT	52.9	4	4	0	100	111	6.17

The data points of RAPD and ISSR were combined for generating similarity index and dendrogram to discover the pattern of molecular divergence. Dendrogram showed two major group formation; in which group I comprised of single genotype J-2296 (R line) whereas, group II consisted of remaining 99 genotypes grouped in three sub-clusters (Fig. 1). The sub-cluster I consists of totally 44 genotypes of which 22 were B lines viz., 261-BCS-19, JMSB20155, 227-BCS-19, 229-BCS-19, 253-BCS-19, 239-BCS-19, JMSB20173, JMSB20175, JMSB20192, 255-BCS-19, 263-BCS-19, 235-BCS-19, JMSB20101, JMSB20174, JMSB20192, 233-BCS-19, 257-BCS-19, 271-BCS-19, JMSB20156, 223-BCS-19, 247-BCS-19, JMSB101; and 22 were R lines viz., J-2596, J-2585, J-2571, J-2576, J-2579, J-2587, J-2589, J-2588, J-2593, J-2467, J-2372, J-2627, J-2517, J-2454, J-2598, J-2500, J-2526, J-2602, J-2566, J-2584, J-104, J-2532. The sub-cluster II consisted of 42 genotypes of which 22 were B lines viz., JMSB20158, 217-BCS-19, 215BCS-19, JMSB20159, JMSB20161, JMSB20181, 243-BCS-19, 249-BCS-19, JMSB20193, 237-BCS-19, 265-BCS-19, JMSB20152, JMSB20154, JMSB20171, JMSB20172, 245-BCS-19, 251-BCS-19, 259-BCS-19, JMSB20102, JMSB20111, JMSB20151, JMSB20143 and 18 were R lines viz., J-2563, J-2619, J-2590, J-2591, J-2569, J-2594, J-2583, , J-2606, J-2480, J-2539, J-2565, J-2340, J-2595, J-2599, J-2607, J-2611, J-2405, J-2597. The sub-cluster III consisted of 12 genotypes represented by 4 B lines i.e., 219-BCS-19, 267-BCS-19, 221-BCS-19, 231-BCS-19 and 08 R lines i.e., J-2582, J-2603, J-2580, J-2604, J-2556, J-2562, J-2552, J-2553.

The similarity coefficient ranged from 0.5 to 3.0; depicting high genetic variation among the lines. Clustering pattern clearly showed that the genotypes are highly diverse from each other; R line J-2296 which formed a single cluster was most diverse in the entire set, showing its importance for exploiting for hybrid breeding by recombining with respective male sterile line of the B lines under study. Markers based heterosis prediction has also been done in many crops like rice, maize, wheat, sorghum, brassica, faba bean, cotton, pepper, chickpea (Passoupathy *et al.,* 2015). Morphological variation is also important to be assessed along with molecular diversity for making precise assumption of heterosis; but past studies have shown phenotypic selection of diverse parents are not always indicative to deliver heterosis in sorghum and no correlation was observed (Rani and Rao, 2009) whereas it was positive in pigeon pea (Pandey *et al.,* 2015). The information generated is very much useful for developing heterotic group based on the information of specific combining ability and general combining ability of these B and

Table 3. Brief information of the RAPD and ISSR band profiles generated

Parameter	RAPD	ISSR
Number of primers used	20	20
Total number of polymorphic bands	162	119
Total number of monomorphic bands	59	22
Total number of bands	221	134
Total number of bands amplified	2046	1994
Average Percentage of polymorphism (%)	72.96	85.02
Average number of bands/primer	7.3	6.7
Average number of polymorphic bands/ primer	5.4	5.95
Average Resolving power	8.89	7.9

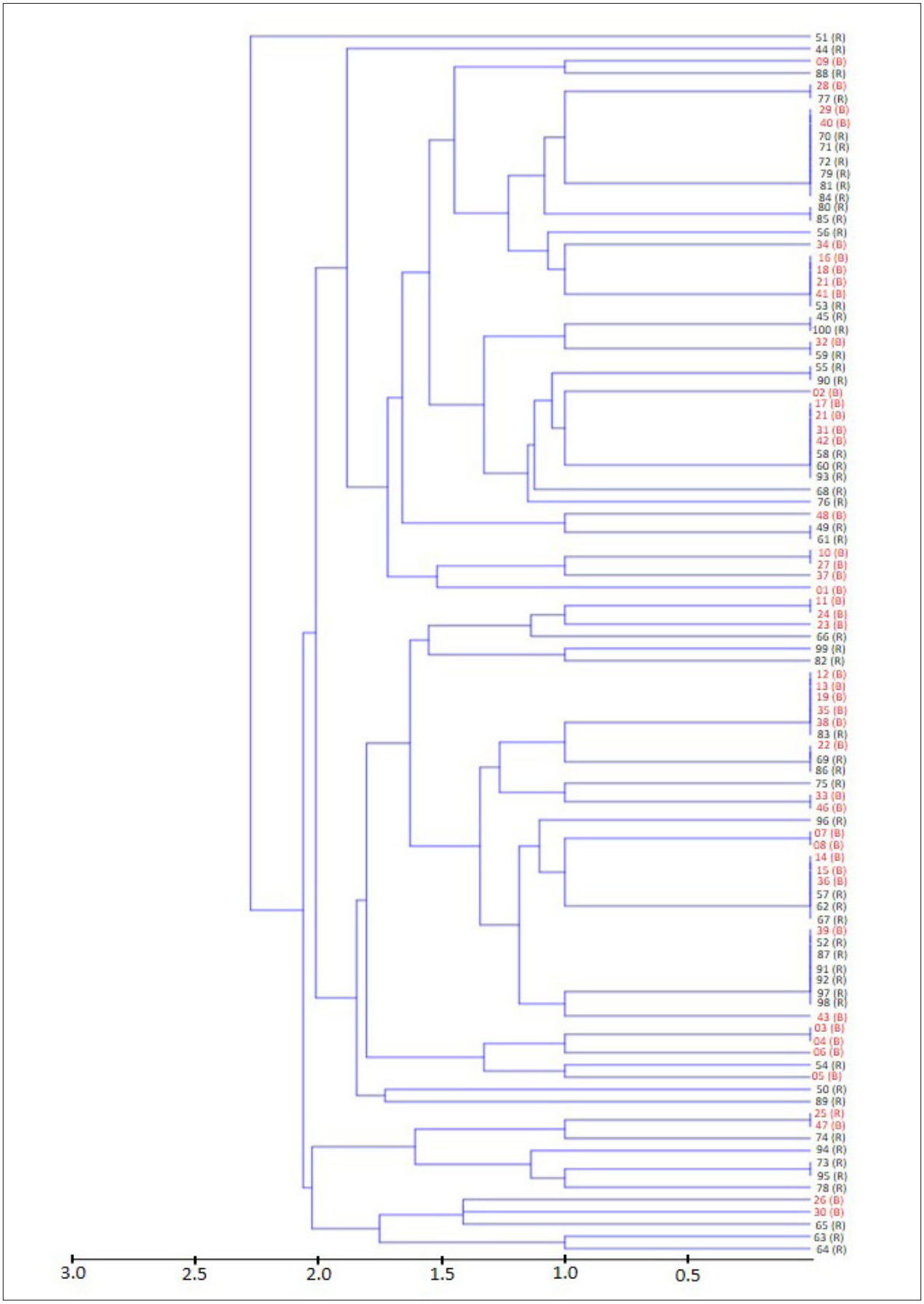

Fig. 1. Genetic similarity index based dendrogram (Genotype number- Black font: R Lines; Red font: B Lines) of 100 Pearl millet genotypes.

R lines. These lines can be further studied for phenotypic diversity and combining ability to make a diverse gene pool for parental selection to undertake hybrid development.

Conclusion: The set of maintainer (B) line and restorer (R) lines are diverse at molecular level. The R line J-2296 was found to be most diverse in the entire group suggesting its intensive use in hybrid development and R line improvement. The classification of genotypes was random to specificity was observed for clubbing of B line or R lines separately. The three sub-clusters formed are offering opportunity to select B line and R lines for inter-mating to exploit heterosis.

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