Morpho-biochemical characterization and molecular marker based genetic diversity of pearl millet [*Pennisetum glaucum* (L.) R. Br.]

Darshanaben F. Gunguniya [1], **Sushil Kumar** [Corresp., 1], **Mukesh P. Patel** [2], **Amar A. Sakure** [1], **Rumit Patel** [1], **Dileep Kumar** [3], **Vikas Khandelwal** [4]

[1] Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat, India

[2] Agriculture and Horticulture Research Station, Anand Agricultural University, Khambholaj, Gujarat, India

[3] Micronutrient Research Centre, Anand Agricultural University, Anand, Gujarat, India

[4] Plant Breeding, ICAR-All India Coordinated Research Project on Pearl Millet, Mandor, Rajasthan, India

Corresponding Author: Sushil Kumar
Email address: sushil254386@yahoo.com

Pearl millet is a key food for millions living the semi-arid and arid regions. The poor's diet contains more pearl millet than other grains. The genetic diversity existing in the pearl millet germplasm can be used to improve the micronutrient content and grain yield. Effective and organized exploitation of diversity at morphological and DNA levels is the strategy for any crop improvement program. In this study, the genetic diversity of 48 pearl millet genotypes was evaluated for eight morphological traits and eleven biochemical characters. All genotypes were also characterized using twelve SSR and six SRAP markers to evaluate genetic diversity. The significant mean difference between morphological and biochemical traits were detected. The productive tillers per plant varied from 2.65 to 7.60 with a mean of 4.80. The grain yield of genotypes varied more than 3x from 15.85 g (ICMR 07222) to 56.75 g (Nandi 75) with an average of 29.54 g per plant. Higher levels of protein, iron, and zinc contents were found to be present in ICMR 12555 (20.6%), ICMR 08666 (77.38 ppm), and IC 139900 (55.48 ppm), respectively, during the experiment. Substantial variability was observed for grain calcium as it ranged from 100.00 ppm (ICMR 10222) to 256.00 ppm (ICMR 12888). The top eight nutrient-dense genotypes flowered in 34–74 days and had 5.71–9.39 g 1000 grain weight. Genotype ICMR 08666 was superior for Fe, Zn, K and P. The inter-genotype similarity coefficient at the genetic level, generated using DNA markers, ranged from 0.616 to 0.877 with a mean of 0.743. A combination of morpho-biochemical traits and DNA markers based diversity may help to differentiate the genotypes and diverse genotypes can be used in breeding programs to improve the mineral content in pearl millet.

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4 Dileep Kumar[3], Vikas Khandelwal[4]

5 [1]Department of Agricultural Biotechnology, Anand Agricultural University, Anand, 388
6 110, India

7 [2]Agriculture and Horticulture Research Station, Anand Agricultural University, Khambhola –
8 388 330, India

9 [3]Micronutrient Research Centre, Anand Agricultural University, Anand 388 110, India

10 [4]ICAR-All India Coordinated Research Project on Pearl Millet, Mandor, Jodhpur - 342 304,
11 India

12 *Corresponding author's email: sushil254386@yahoo.com

14 **Abstract**

15 Pearl millet is a key food for millions living the semi-arid and arid regions. The poor's diet
16 contains more pearl millet than other grains. The genetic diversity existing in the pearl millet
17 germplasm can be used to improve the micronutrient content and grain yield. Effective and
18 organized exploitation of diversity at morphological and DNA levels is the strategy for any crop
19 improvement program. In this study, the genetic diversity of 48 pearl millet genotypes was
20 evaluated for eight morphological traits and eleven biochemical characters. All genotypes were
21 also characterized using twelve SSR and six SRAP markers to evaluate genetic diversity. The
22 significant mean difference between morphological and biochemical traits were detected. The
23 productive tillers per plant varied from 2.65 to 7.60 with a mean of 4.80. The grain yield of
24 genotypes varied more than 3x from 15.85 g (ICMR 07222) to 56.75 g (Nandi 75) with an
25 average of 29.54 g per plant. Higher levels of protein, iron, and zinc contents were found to be
26 present in ICMR 12555 (20.6%), ICMR 08666 (77.38 ppm), and IC 139900 (55.48 ppm),
27 respectively, during the experiment. Substantial variability was observed for grain calcium as it
28 ranged from 100.00 ppm (ICMR 10222) to 256.00 ppm (ICMR 12888). The top eight nutrient-
29 dense genotypes flowered in 34–74 days and had 5.71–9.39 g 1000 grain weight. Genotype
30 ICMR 08666 was superior for Fe, Zn, K and P. The inter-genotype similarity coefficient at the
31 genetic level, generated using DNA markers, ranged from 0.616 to 0.877 with a mean of 0.743.

32 A combination of morpho-biochemical traits and DNA markers based diversity may help to
33 differentiate the genotypes and diverse genotypes can be used in breeding programs to improve
34 the mineral content in pearl millet.

35 **Key words:** Genetic diversity, Germplasm, Grain minerals, Pearl millet, Variability, Yield

36 **1. Introduction**

37 Pearl millet [*Pennisetum glaucum*(L.)] is a small-seeded C4 plant of the Poaceae family. The 1.7
38 GB genome of this crop is accommodated by 2n=2x=14 chromosomes. Compared to other
39 cereals like wheat and rice, it can withstand effectively in drought, nutrient-depleted soil, and
40 hot environmental conditions of the hostile deserts of India and Africa. This hardy nature makes
41 pearl millet resilient to harsher climatic conditions consequently it is cultivated in marginal
42 environments of arid and semi-arid tropical regions of India and south east Asia, sub-Saharan
43 Africa and much of southern and eastern Africa (Ramya et al., 2018). Pearl millet is a key food

44 for millions living the semi-arid and arid regions. The grain is mainly consumed as human food
45 while biological yield is used as livestock feed. Pearl millet is a primary food for mankind living
46 in dryland agriculture regions.

47 Pearl millet accounts for circa 50% of the total global millet production. It is grown on >28
48 million ha, mainly in Africa and India. India is the world's largest producer of millets, harvesting
49 11 million tonnes annually, or around 36% of global production. In 2020, India harvested 8.61
50 million tonnes of pearl millet grains from a 6.93 million ha area with 1,243 kg/ha of productivity
51 (Directorate of Millets Development, AICPMIP, 2020).

52 In any breeding strategy, variation continues to be the key to success. Pearl millet shows
53 abundant phenotypic variability for most of the quantitative traits like flowering time, ear head
54 length, grain characteristics, tolerance to various (a)biotic stresses as well as nutritional quality
55 (Bhattacharjee et al., 2007). Effective and logical utilization of this diversity is vital to any
56 breeding program (Allard et al., 1960). Exploiting this genetic diversity in the pearl millet
57 population may allow the improvement of micronutrient density in grain and grain yield.

Micronutrient insufficiency has emerged as a global problem, particularly for those living in underdeveloped nations and consuming carbohydrate-rich cereal-based diets. This deficiency can be managed with pearl millet, a nutritious cereal (Kumar et al., 2016). Among all coarse cereals, pearl millet grains are dense in minerals like iron (Fe) and zinc (Zn) concentration and essential amino acids. The protein in pearl millet ranges between 9 - 21%, which is higher than sorghum (10.4%), rice (6.8%), and maize (4.7%) (Kaur et al., 2014). The grains of pearl millet are gluten-free and have a low glycemic index due to their high fiber content. The provitamin-A enriched grains are also a richer source of fat (5-7 g/100 g) but are scarce in vitamins B and C (Gopalan et al., 2003). Pearl millet grain is encased in a tough fibrous seed that contains a variable amount of inhibitory factors like phytic acid and polyphenols (Arora et al., 2003). But these factors can be reduced through various approaches like soaking, fermentation, blanching, and roasting (Kaushik and Grewal, 2017) up to a certain extent only. Moreover, the presence of anti-nutrient factors like saponins, tannins and phytic acid which can reduce nutrient utilization or food uptake hinders the biofortification in millet.

To improve the nutritional quality and diminish the anti-nutritional factors of pearl millet through any breeding approach, knowledge about the variability for mineral content, anti-nutritional factors and their relation with yield is a prerequisite. Accumulation of both micro- and anti-nutrients in seeds is a complex mechanism containing numerous genes and affected by the environment (Anuradha et al., 2017). Due to the confounding effect of the environment, similar genotypes would have different phenotypes due to environmental variability. Therefore, it is hard to equate morpho-biochemical and genetic variability. In contrast, molecular markers reflect the authentic genetic variability and relationships among accessions than phenotypic markers (Glaszmannet al.2010). In pearl millet, microsatellite, single nucleotide polymorphisms (SNP) and restriction fragment length polymorphism (RFLP) markers have been applied to create linkage maps followed by quantitative trait loci mapping and germplasm characterization (Kumar et al., 2020). The density and genome coverage can be improved by the combination of various markers. In pearl millet, no report is available on the deployment of sequence-related amplified polymorphism (SRAP) markers for genetic diversity assessment though SRAP has been used for linkage map development by Pedraza-Garcia et al. (2010). Therefore, in the current experiment, both SRAP and microsatellite markers have been used to expose the genetic diversity. SRAP markers are dominant markers that target genomic coding sequences and have

89   been employed for genetic diversity assessment (Li and Quiros, 2001). The current study was

90   created to analyse the natural variability for grain mineral and anti-nutrient content in grain as

91   well as molecular diversity in pearl millet germplasm with the objective of improving the

92   nutritional quality and food safety of pearl millet as well as expanding understanding in this area.

93   **Materials and methods**

94   The field trial was done in a randomized complete block design (RCBD) with two replications.

95   The inter- and intra-row distance was 60 and 15 cm, respectively. The recommendations for crop

96   management practices were followed for uniform plant growth and a healthy crop stand. The

97   seeds were sown on February 2021. A total of 48 pearl millet genotypes were used for the study

98   (Supp Table 1).

99   *1.1.   Morphological characters*

100 The panicles were covered with glassine bags to prevent cross-pollination by outside pollen and

101 to collect self-seeds. For phenotyping of grain based traits, physiologically mature panicles were

102 collected, dried under sunlight, and then manually threshed in bulk.

103 The experimental material was evaluated for eight morphological traits viz., days to 50%

104 flowering, plant height, panicle diameter (PD, cm), panicle length (PL, cm), number of

105 productive tillers, grain yield, days to maturity, and 1000 grain weight. Except for days to 50%

106 flowering and days to maturity, which were recorded on a plot basis during the study, data on the

107 above traits were collected from randomly tagged five competitive plants in each genotype in

108 both replications. PD was measured with Vernier calipers.

109 *1.2. Biochemical characters*

110 Before biochemical analysis, grains were cleaned followed by hot air oven drying (80°C for 24

111 h). Dried grains (10 g) were powdered manually. For mineral analysis, 0.5 g flour was processed

112 in 11 mL of nitric acid (69%) and 1 mL of H_2O_2. The digestions were carried out in HVT50

113 vessels using rotor 12HVT50 in Multiwave GO/ Multiwave GO plus (Anton Paar GmbH,

114 Austria). For microwave digestion the initial temperature was kept at 180°C with a ramp of 20

115 minutes and a hold period of 12 minutes. While the second round of digestion was performed

116 with a ramp time of 10 mins at 70°C temperature and a holding time of 5 mins. Inductively

117 Coupled Plasma Optical Emission Spectrometry (ICP-OES (model 7000DV of make: Perkin

118 Elmer, made in USA with wintab32 software ver. 5.1) was used to determine the mineral

119 content [iron (Fe), zinc (Zn), Calcium (Ca), copper (Cu), and manganese (Mn)] in grain after

120 diluting the digested mixture to a volume of 50 ml using distilled water. The flow rate in a

121 peristaltic pump was 1.5ml per min. From the acid extract, potassium (K) content was quantified

122 using a flame photometer (Jackson, 1973), while the vanadate-molybdate method of Jackson

123 (1973) was used to estimate the phosphorus (P). Total phenols were estimated using the Folin-

124 Ciocalteau reagent as Malik and Singh (1980) and reading was measured at 730 nm using a

125 spectrophotometer. Soxhlet extraction was performed to estimate the crude oil content, and

126 semimicro-Kjeldahl was employed to determine the crude protein content.

127 *1.3.   Molecular marker study*

128 Genomic DNA was extracted from tender leaves as per Mace et al (2003). Genotyping was done

129 using simple sequence repeat (SSR) and sequence-related amplified polymorphism (SRAP)

130 markers. For SSR marker profiling, markers from the PMES series (Zala et al., 2017) were

131 amplified in SensoQuest Thermocycler (Germany). The SSR-PCR reaction conditions were as

132 follows: 94°C (initial denaturation) for 5 min., followed by 35 cycles of 94°C for 45 sec, X°C

133 (primer specific) for 45 sec, 72°C for 45sec, and 72°C for 7 mins (final extension) . The

134 SRAP amplification was as follows: 94 °C (initial denaturation) for 5 mins., followed by 5 cycles

135 of 94 °C for 30 s, 35°C for 45 s, and 72°C for 90 s, followed by 35 cycles of 94°C for 30 s, X°C

136 (primer specific) for 45 s, 72 °C for 60 s and 72 °C for 10 min (final extension). An agarose gel

137 (3%) was used to resolve PCR products.

138 *1.4.   Statistical analysis*

139 The mean value of traits was figured out, and analysis of variance (ANOVA) was performed in

140 accordance with Panse and Sukhatme (1978) in Microsoft Excel 2013. A phenotypic trait-based

141 dissimilarity matrix was constructed using Manhattan coefficients with Numerical Taxonomy

142 and Multivariate Analysis System (NTSYSpc 2.0;Rohlf 1998). The amplified products of SSR

143 and SRAP markers were scored in 1 (presence) and 0 (absence) fashion. Polymorphism

144 information content (PIC), Multiplex ratio (MR), effective multiplex ratio (EMR) marker index

145 (MI) and resolving power (Rp) value were estimated following Sharma et al. (2016) in Microsoft

146   Excel 2013. In NTSYSpc 2.0, The SIMQUAL program used Jaccard's similarity (J) coefficient

147   to compute the genetic similarity between genotypes, SAHN clustering method was used to

148   construct the unweighted pair group method with arithmetic mean (UPGMA) dendrogram

149   (Sneath and Sokal, 1973).

150   **3.   Results and discussion**

151   The ANOVA resulted that genotypic variations were significant at a 5% level of probability for

152   all the traits, showing ample genetic diversity among the genotypes under study (Table 1). This

153   also suggested that there is sufficient scope to select superior breeding material which can be

154   exploited in pearl millet breeding programs.

155   *3.1.   Character variance analysis*

156   *3.1.1. Morphological parameters*

157   Early flowering is a desirable trait for pearl millet as it is a crop of semi-arid and arid regions.

158   Earliness becomes an important trait in areas where scanty and erratic rains aggravate the

159   moisture stress condition during the growth stage of the crop and leads to post-flowering

160   moisture stress (Yadav et al., 2011). In the current study, though, the population mean for days

161   to 50% flowering was 54.69 days but the days to 50% flowering ranged from 34 (IC 370523) to

162   77 days (ICMR 07999). Earlier literature also recorded similar values for days to 50% flowering

163   for example 49.06 days by Govindaraj et al. (2011), 53.10 days by Sonali et al. (2019) and 55.61

164   days by Pallavi et al. (2020). PH is an important trait that governs tradeoffs between competition

165   and resource distribution, which is decisive for productivity (He et al., 2021). Semi-dwarf

166   genotypes are better than their tall counterparts because of their reduced lodging vulnerability

167   and better response to nitrogen Azhaguvel et al. (2003). In the present experiment, PH ranged

168   from 110.10 cm (ICMR 06555) to 205.35 cm (IC 332715) with an average of 149.42 cm. The

169   results indicated that most of the studied genotypes are semi-dwarf in nature and with better

170   management dwarfism supports the grain yield.

171   Panicle size (length and diameter) are two important traits that have direct positive correlations

172   with grain yield in pearl millet (Vengadessan et al., 2013). Hence, the improvement of sink-size

173   linked traits is a key objective for pearl millet improvement programs. PL in the present study

174   ranged from 15.55 cm (ICMR 11888) to 38.05 cm (IC-332716) with an average of 24.03 while

175   the diameter ranged from 1.03 cm (ICMR 10999) to 2.15 cm (ICMR 09333) with an average of

176   1.53 cm. Abubakar et al. (2019) observed a similar range and mean in pearl millet (2.26 cm) for

177   panicle diameter. Similarly, results for PL are comparable with previous reports (Sharma et al.,

178   2018, Rani et al., 2019). The number of productive tillers per plant varied from 2.65 (ICMR

179   08222, ICMR 11999) to 7.60 (IC 370523) with an average of 4.80 (Table 2). According to Sile et

180   al. (2004), non-tillering millet genotypes produced bold seeds having TGW >10gm than the

181   genotype that produced tillers. Similarly, Maman et al. (2004) also reported that, a reduction in

182   productive tillers from 10 to 3 or 5 improved seed yields by 15-30%. Yadav et al (2021) reported

183   that private-sector hybrids are generally have less effective tillers/plant. But still, farmers in

184   drought-prone areas prefer high tillering hybrids because tillering is a strategy of adaptation to

185   intermittent drought spells (Yadav et al., 2016).

186   In cereal breeding, yield, a complex trait, is one of the supreme traits which is influenced by

187   several associated traits. The grain yield of genotypes varied more than 3x from 15.85 g (ICMR

188   07222) to 56.75 g (Nandi 75) with an average of 29.54 g per plant. Large variability was also

189   observed for1000 grain weight (TGW) which is determined by the form, size and density of the

190   grain and these are directly related to total grain yield. TGW ranged from 4.93 g (ICMR 06888)

191   to 10.45 g (ICMR 06555) with an average of 7.14 g. A diversity assessment of 21,594 pearl

192   millet genotypes from 50 nations revealed huge variability for the TGW (1.5 to 21.3 g)

193   (Upadhyaya et al., 2007). Three-fold variability for TGW (6-16 g) was earlier recorded by Pujar

194   et al (2018).

195   *3.1.2. Biochemical parameters*

196   Compared to other main cereal crops, pearl millet yields more nutritious grains with protein, Ca,

197   P, Fe, and Zn (Devos et al., 2006). Currently, the commercially grown varieties/hybrids of pearl

198   millet produce grains with an average Fe and Zn content of 42 and 32 ppm (parts per million),

199   respectively (Rai et al. 2016). However, a much wider variability for these micro-nutrients has

200   been reported in germplasm collections (Rai et al. 2014). Fe is an essential element for blood

201   production and for the growth and development of the body. Zn is essential for the development

202   of a strong immune system. The values of Fe content in the current study ranged from 31.58

203   (ICMR 07777) to 77.38 (ICMR 08666) with an average of 49.69. Zn content ranged from 29.34

204   (ICMR 08111) to 55.48 (IC 139903) with an average of 39.36 (Table 2). A similar mean value

205   was observed by Velu et al. (2007) where grain Fe was 45.50 ppm. In previous studies, values

206 ranging from 45.50-55.73 for grain Fe concentration and from 38.60-46.61 for grain Zn
207 concentration have been reported (Anuradha et al., 2017; Anuradha et al., 2018; Sonali et al.,
208 2019; Yadav et al., 2020).

209 In the human body, fats and carbohydrates metabolism, absorption of Ca, and the control of
210 blood sugar are all impacted by manganese. It is also essential for standard brain/nerve
211 functioning and bone mineral density. The values of Mn content ranged from 7.20 ppm (ICMR
212 07222) to 17.63 ppm (ICMR 08444) with an average of 14.04 ppm. The outcome is in
213 congruence with Anuradha et al. (2017), Kumar et al. (2020a) and Govindaraj et al. (2020a).
214 Similarly, a low value of Mn (8 ppm) was recorded by Oshodi et al. (1999).

215 Ca is very important for the contraction of muscle; the development of strong bones and teeth,
216 blood clotting, the transmission of nerve impulses, and in the regulation heart beats. This is
217 claimed that a high intake of cereal grains increases the chances of Ca deficiency. But this is not
218 true with pearl millet as substantial variability was observed for grain Ca as it ranged from
219 100.00 ppm (ICMR 10222) to 256.00 ppm (ICMR 12888) with an average of 199.31 ppm.
220 Higher variability for Ca (85-249 ppm) was also recorded by Govindaraj et al. (2020b) in pearl
221 millet core collection. In the current study, 50% of the genotypes had high Ca (>200 ppm).

222 Cu is essential for the synthesis of elastin and collagen. It is a key cofactor of many
223 metalloenzymes playing role in metabolism Fe and cellular respiration. In the current study, the
224 grain Cu ranged from 4.92 ppm (IC 332703) to 22.59 ppm (GHB 558) with an average of 9.86
225 ppm. The range of grain Cu in various studies is different like 4.14-15.35 ppm in Anuradha et al.
226 (2018), 4-7 ppm in Govindaraj et al. (2020), 3.19-4.76 ppm in Warrier et al (2020).

227 The transport of water, nutrients, and carbohydrates within plant cells is linked with potassium. It
228 is a crucial mineral for the activation of several enzymes that control the synthesis of protein,
229 starch, and adenosine triphosphate (ATP) in plants. The K ranged from 1800 ppm (ICMR 07222)
230 to 6000 ppm (ICMR 10999) with an average of 4700 ppm. Large variability for K was also
231 recorded in 122 commercial pearl millet cultivars (3675–5375ppm; Govindaraj et al. 2020a) and
232 core collection (3667-5133 ppm; Govindaraj et al. 2020b).

233 The body needs P to produce protein for the development, upkeep, and repair of cells and tissues.
234 Additionally, it participates in the production of ATP. The values of P content ranged from 2200

235   (IC 139900) to 3600 ppm (ICMR 08666) with an average of 3112 ppm. ICMR 06555 was

236   statistically at par with IC 139900.

237   Pearl millet is also a promising source of protein. Studies indicated that protein in pearl millet is

238   circa 11.8 %, which is better than rice (8.6 %), and maize (9.2 %) and comparable with sorghum

239   (10.7 %). Moreover, pearl millet grain is enriched with glutamate which is a precursor of γ-

240   aminobutyric acid (GABA) (Tomar et al., 2021). In the current study the protein content ranged

241   from 8.26% (IC 332716) to 20.06% (ICMR 12555) with an average of 13.73%. ICMR 07444

242   (9.89%) was statistically at par with IC 332716. The study of Pujar et al. (2020) reported grain

243   protein content variation between 6 - 18%, with a mean of 11%. The augmentation of pearl

244   millet in daily food can reduce the risk of protein malnutrition in an economical way. Moreover,

245   protein extracted from pearl millet can be exploited to design protein-enriched functional foods.

246   The lipid content ranged from 2.72% (ICMR 06999) to 6.95% (ICMR 08444) with an average of

247   4.68%. A comparable range and mean were observed by Arulselvi et al. (2007; 5.12%), Abdalla

248   et al. (2007; 2.70-7.10%) and Tomar et al. (2021; 5.24-9.99). The lipid content in pearl millet

249   ranges from 1.5 to 6.8% which is higher sorghum and other millets (Hassan et al., 2021).

250   Though, the high lipids have been documented as possible causes for the rancidity of millet

251   flour. However, the shelf life of flour can be increased by hydrothermal treatment, irradiation,

252   cooling storage, or a combination of more than one technology (Goyal and Chugh, 2017).

253   The metal-chelating ability of phytic acid makes it is an antinutritional phytochemical as it

254   declines the bio-availability of ions like Mn, Ca, Mg, Fe and Zn (Marathe et al., 2018). In the

255   current study, the phytate ranged from 201.5 mg/100g (ICMR 08111) to 542.50 mg/100g (GHB

256   558) with an average of 282.39. Abdalla et al. (2007) also recorded a similar range from 354-795

257   mg/100g of phytate. Gabaza et al. (2018) reported that phytate in pearl millet grains ranges

258   between 580 mg/100g to 1380 mg/100g which is similar to sorghum and maize. The range of

259   phytate in the current study is supported by the result of Pushparaj and Urooj (2014) in Indian

260   cultivars where it was between 0.26 - 0.99 g/100 g. The result suggested that phytic acid content

261   in pearl millet grain is significantly lower than in rice (0.68-1.03%; Liu, 2005), oat (0.5–1.2%;

262   Peterson, 2001), soybean (1.0–2.22%; Lolas et al. 1976) and wheat (0.2 - 2.9%; Gupta et al.

263   2015) Hence, regular consumption will possibly not hamper the bioavailability of minerals.

264 Polyphenols have many health benefits as having antioxidant activity. Moreover, phytic acid is
265 considered to be beneficial in dropping cholesterol and reducing cancer risk. The values of total
266 phenolic acid ranged from 44.41mg/100g (Nandi 75) to 75.16mg/100g (ICMR 12555) with an
267 average of 60.26mg/100g. Higher phenol content in grain makes pearl millet a good food to
268 maintain the redox potential of cells and to quench the ROS species. Phenolic may be
269 particularly important in the treatment of postprandial hyperglycemia since it has been
270 documented that it reduces intestinal -glucosidase and pancreatic -amylase (Shobana et al. 2009).

271 *3.2.   Nutrient-dense genotypes*

272 Genotypes dense in multiple nutrients can directly be released as a variety after evaluating their
273 yield performance over the locations for multiple years. Such genotypes can be exploited in a
274 hybridization program. In the current study, the top eight nutrient-dense genotypes flowered in
275 34–74 days and had 5.71–9.39 g TGW (Table 3). Top genotypes had Fe content of 61.07-77.38
276 (ICMR 08666) ppm, Zn content of 45.11-55.48 (IC 139900) ppm, Mn content of 16.2-17.63
277 (ICMR 08444) ppm, Ca content of 230.5-256 (ICMR 12888) ppm, Cu content of14.32-22.59
278 (GHB 558) ppm, K content of 52.3-60.15(ICMR 10999) ppm, and P content of 33.28-36.72
279 (ICMR 08666). IC 139900 was superior for both Fe (71.22 ppm) and Zn (55.48 ppm). Genotype
280 ICMR 08666 was dense for Fe, Zn, K and P. Out of eight high-Fe genotypes, only two genotypes
281 had > 75 ppm. Thus current experiment also identified the best genotypes that had a higher
282 content of multiple nutrients. Earlier, Govindraj et al (2020) also identified genotypes having a
283 high content of multiple nutrients.

284 *3.3.   Phenotypic diversity analysis*

285 Phenotypic diversity is important for pearl millet breeding. The interactions between the genome
286 and all of its growing micro- and mega-environments lead to the phenotype of the plant (Fasoula
287 et al., 2020). The mean value of each trait was used to generate the Manhattan dissimilarity
288 coefficient and dendrogram (Sokal and Michener, 1958). The genotypes were divided into nine
289 major clusters based on the Manhattan dissimilarity coefficient. Earlier, Shashibhushan et al
290 (2022) also generated eight clusters of 40 pearl millet genotypes using phenotypic data (Figure
291 1). In current study, the average dissimilarity value among genotypes was calculated to be 0.16,
292 demonstrating modest phenotypic variability (Table 4). The dissimilarity between genotypes

293 ranged from 0.08 (IC 139899 and ICMR 07888) to 0.27 (Nandi 75 and ICMR 07222) for the
294 respective pair of genotypes.

295 Cluster I comprise seven genotypes, characterized by high values of DFF, days to maturity, lipid,
296 potassium and low values of NPT. Cluster II consists of 25 genotypes. Cluster III contains four
297 genotypes, namely ICMR 10222, Nandi 75, ICMR 12111, and ICMR 10888. This cluster is
298 characterized by more GY, days to maturity and TGW. Cluster IV has four genotypes, namely
299 ICMR 08999, ICMR 11888, ICMR 139900 and ICMR 07777. This cluster is characterized by
300 more lipid content, Mn content, and days to maturity. Cluster V has three genotypes (IC 332716,
301 GHB 558, ICMR 07444) which are characterized by low values of head diameter and lipid
302 content. Cluster VI consists of two genotypes (ICMR 08111 and ICMR 12333). This cluster is
303 characterized by more productive tillers per plant with low content of Zn. Cluster VII has only
304 one genotype (ICMR 12555) which has high values for characters like days to maturity, protein
305 content, Mn content and TGW (Table 4).

306 *3.4.   Molecular marker based diversity*

307 Forty-eight genotypes of pearl millet were analyzed using SSRs and SRAP markers (Table 5).
308 During the experiment, a total of 50 SSR markers were screened for amplification. Out of 50
309 markers, 30 (60%) primers showed amplification. Of these 30 SSR markers, 12 (37.5%) markers
310 were found polymorphic. These 12 polymorphic SSRs markers generated 65 amplicons. The
311 molecular weight of the amplicon ranged from 85bp (PMES 190) to 292bp (PMES 171). In
312 previous reports with PMES-series SSR markers, Zala et al. (2017) recorded amplicon size from
313 101 to 285 bp. The number of polymorphic bands/amplicons per SSR marker ranged from 2 to
314 13, with a mean of 6.71. All SSR amplicons were found polymorphic. The PIC value, the
315 informativeness of a primer, for each marker was computed for the estimation of marker allelic
316 variation considering the allele frequencies in studied genotypes. The mean PIC of SSR markers
317 was 0.28 though it varied from 0.132 (PMES 171) to 0.499 (PMES 173). This range was
318 comparable with Zala et al. (2017) where PIC was between 0.188 - 0.375.

319 The Rp was estimated considering the proportion of genotypes containing the amplicon. The
320 primer that might best differentiate the cultivar can easily be identified by the value of the Rp
321 and PIC. In the current study, Rp varied from 1.625 (PMES 162) to 2.375 (PMES 168), with an

322   average of 1.98. Mean Rp was between 0.154 (PMES 171) to 1.000 (PMES 173). The PI ranged

323   from 0.998 (PMES 173) to 1.963 (PMES 168), though the mean PI value was 1.420. MI is

324   considered to be an inclusive measure of the efficiency to detect polymorphism. The SSR MI

325   was 19.092.

326   In the case of the SRAP markers, of 25 SRAP, 6 (24%) were polymorphic. The polymorphic

327   SRAPs amplified 119 amplicons. The product size for SRAPs ranged from 94 (Em2+Me2) to

328   1357 bp (Em6+Me3). The polymorphic bands ranged from 10 (Em6+Me3) to 34 (Em2+Me2),

329   with a mean of 21.67. Liu et al. (2008) observed a polymorphic band detected with each ranging

330   from 6 to 17, with an average of 11.76. Bhatt et al. (2017) had a band size from 120 to 500 bp in

331   cumin.This suggested that in different crops SRAP amplicon size will be highly variable. PIC

332   oscillated from 0.224 (Em5+Me4) - 0.324 (Em6+Me2), with an average of 0.26. Bhatt et al.

333   (2017) reported a PIC value (0.34).

334   The mean PI of SRAP markers was 5.69, through the maximum PI was for Em6+Me2 (8.108)

335   and the lowest value for Em6+Me3 (2.335). Rp ranged from 4.625 (Em5+Me4) to 13.250

336   (Em6+Me2), with an average value of 8.27. Mean Rp was between 0.272 (Em5+Me4) - 0.542

337   (Em6+Me3) with an average of 0.40. Liu et al. (2008) stated higher RP values ranged from 2.229

338   to 8.457 with an average of 4.927. The Fraction of polymorphism, MR, EMR and MI for SRAPs

339   are 1.00, 10.00, 10.00, and 15.65, respectively (Table 6).

340   *3.5.   Inter-Genotype Genetic Relationship*

341   Forty-eight pearl millet genotypes were divided into seven major groups by the dendrogram

342   created using pooled data from SSR and SRAP markers based on Jaccard's similarity matrix.

343   Cluster I, II, III, IV, V, VI and VII had 1, 1, 11, 8, 3, 22, and 2 genotypes, respectively (Figure

344   2). In the study of Nehra et al. (2017), with SSR markers, 49 accessions were clustered into eight

345   core clusters. Kumar et al. (2020b) alienated 18 lines into three clusters in pearl millet using 74

346   SSRs. In the current study, the inter-accession genetic coefficient of similarity ranged from 0.616

347   to 0.877 while the average similarity was 0.743. ICMR 098888 and GHB 905 has a genetic

348   distance (0.384) indicative that both genotypes are having moderate genetic difference level and

349   can be crossed to create a bi-parental mapping population. The minimum genetic distance

350   (0.123) was between IC 139899 and IC 332727, demonstrating that these accessions have more

351 similarity in SSR locus. Moreover, based on diversity results, breeders can select diverse
352 genotypes for combining ability and heterosis analysis for traits studied in the current study.

353 **4. Conclusion**

354 The genetic diversity for morphological and grain biochemical traits, an outcome of natural
355 selection with the cross-pollination nature of pearl millet, was revealed by analysis of variance.
356 Variability for grain micronutrient content was found greater with a wide range in the
357 population. Genotypes namely ICMR 08666 and IC 139903 were superior for Fe and Zn content,
358 respectively. Genotype ICMR 08666 was also found promising for Zn, K and P content and can
359 further be utilized for genetic biofortification. In the present study, phenotypic diversity analysis
360 grouped all genotypes into nine different clusters. Among all clusters, three clusters had only
361 single genotype with better phenotypic value for most of the grain biochemical parameters. But
362 phenotype is a total outcome of the genotype and its interaction with the environment. Genetic
363 markers are found effective in this study, they help to identify ICMR 098888 and GHB 905 as
364 diverse genotypes for making a bi-parental mapping population.

365 **5. Conflict of Interest**
366 All authors declared that the research was conducted in the absence of any commercial or
367 financial relationship that could be construed as a potential conflict of interest.

368 **6. Authors' Contribution**
369 **SK, MPP, AAS & VK:** Conceptualization & Designing the experiment, **DFG, RP & DK**:
370 performed the experiments, **RP & SK**: analyzing the data, **VK:** Contribution of experimental
371 material, **DFG, SK & RP**: Prepared the figures and tables, **SK & AAS**: Critically revised the
372 manuscript for important content,

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378

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585   365.

Analysis of variance (ANOVA) of studied traits in pearl millet

1   Table 1. Analysis of variance (ANOVA) of studied traits in pearl millet

Trait	Source of variation and Mean squares		
	Replication (df = 1)	Genotypes (df = 47)	Error (df = 47)
Days to 50% flowering	0.167	322.311*	5.954
Plant height	66.833	917.408*	62.211
Head diameter	0.027	0.159*	0.027
Panicle length	1.927	46.955*	3.880
Productive tillers per plant	0.027	3.712*	0.217
Grain yield per plant	11.003	203.764*	30.441
Days to maturity	2.344	9.292*	3.471
1000 grain weight	0.000176	2.863*	0.104
Protein content	2.004	10.363*	0.705
Lipid content	0.062	3.851*	0.052
Iron content	14.015	276.360*	16.714
Zinc content	41.12	79.610*	13.100
Manganese content	2.154	11.232*	1.576
Calcium content	661.5	2061.205*	332.691
Copper content	0.218	43.735*	0.551
Potassium content	57.722	102.837*	14.517
Phosphorus content	5.782	12.004*	5.093
Phytate content	895.482	8396.470*	541.246
Total phenolic acid	26.471	112.792*	36.582

2   *Significant at 5% level of probability

Descriptive statistics for studied traits in pearl millet

S.Em.: Standard error of mean; CD @ 5%: critical difference at 5% level of significance; CV: Coefficient of variance

1   Table 2. Descriptive statistics for studied traits in pearl millet

Trait	Mean	Range	S.Em	CD @ 5%	CV%
Days to 50% flowering	54.69	34.00 (IC-370523) - 77.00 (ICMR 07999)	1.73	4.91	4.46
Plant height (cm)	149.42	110.10 (ICMR 06555) - 205.35 (IC-332715)	5.58	15.87	5.28
Head Diameter (cm)	1.53	1.03 (ICMR 10999) - 2.15 (ICMR 09333)	0.12	0.33	10.75
Panicle length (cm)	24.03	15.55 (ICMR 11888) - 38.05 (IC-332716)	1.39	3.96	8.2
Productive tillers per plant	4.80	2.65(ICMR 08222, ICMR 11999)-7.60(IC-370523)	0.33	0.94	9.7
Grain yield per plant	29.54	15.85 (ICMR 07222) - 56.75 (Nandi 75)	3.9	11.1	18.67
Days to maturity	85.64	80.50 (ICMR 12333) - 89.00(ICMR 08999, ICMR 07777)	5.82	16.57	9.71
1000 grain weight	7.14	4.93 (ICMR 06888) - 10.45 (ICMR 06555)	0.23	0.65	4.51
Protein content (%)	13.73	8.26(AICRP-PM-12)-20.06(ICMR 12555)	0.59	1.68	6.12
Lipid content (%)	4.68	2.72(ICMR 06999)-6.95(ICMR 08444)	0.16	0.46	4.89
Iron content (ppm)	49.69	31.58 (ICMR 07777)-77.38 (ICMR 08666)	2.89	8.22	8.23
Zinc content (ppm)	39.36	29.34 (ICMR 08111)-55.48 (IC 139903)	2.65	7.53	9.5
Manganese content (ppm)	14.04	7.20 (ICMR 07222)-17.63(ICMR 08444)	0.89	2.53	8.94
Calcium content (ppm)	199.31	100.00 (ICMR 10222)–256.00 (ICMR 12888)	12.9	36.69	9.15
Copper content (ppm)	9.86	4.92 (AICRP-PM- 6)-22.59(GHB 558)	0.52	1.49	7.52
Potassium content (ppm)	4798	1800 (ICMR 07222)-6020(ICMR 10999)	2.69	7.67	7.94
Phosphorus content (ppm)	3112	2258(AICRP-PM-62)-3672(ICMR 08666)	1.6	4.54	7.25
Phytate content (mg/100g)	282.39	201.5(ICMR 08111)-542.5(GHB 558)	16.45	46.8	8.24
Total phenolic acid (mg/100g)	60.26	75.16(ICMR 12555)-44.41(Nandi 75)	4.28	12.17	10.04

2   S.Em.: Standard error of mean; CD @ 5%: critical difference at 5% level of significance; CV: Coefficient of variance

Top eight genotypes for different nutritional traits with their agronomic performance

1   Table 3. Top eight genotypes for different nutritional traits with their agronomic performance

Trait	Top 8 genotypes	Range	
		DFF	TGW (g)
Fe	ICMR 08666, ICMR 11888, IC 332727, IC 139900, ICMR 12555, ICMR 06111, ICMR 08333, ICMR 07888	37.00-72.00	5.91-8.45
Zn	IC 139903, ICMR 08999, ICMR 06222, ICMR 12999, ICMR 10999, ICMR 08666, IC 332715, IC 332703	42.00-70.50	5.94-9.39
Mn	ICMR 08444, ICMR 12555, ICMR 09888, ICMR 08333, ICMR 06666, ICMR 12666, ICMR 09222, IC 332703	45.00-74.50	5.91-8.01
Ca	ICMR 12888, ICMR 12777, ICMR 07444, ICMR 09333, ICMR 11777, IC 332703, ICMR 08999, ICMR 08333	35.50-72.50	5.91-8.36
Cu	GHB 558, IC 332715, IC 332716, ICMR 06666, ICMR 07222, ICMR 07444, ICMR 07777, ICMR 10999	42.00-73.50	5.71-8.02
K	GHB 558, GHB 732, GHB 905, ICMR 08666, ICMR 10999, ICMR 12555, ICMR 12666, ICMR 12777	43.50-74.00	6.16-8.99
P	ICMR 08666, ICMR 06888, ICMR 06111, IC 370523, IC 332727, IC 139899, Nandi 75, ICMR 06666	34.00-73.50	4.93-9.34

2   DFF: days to 50% flowering; TGW: 1000-grain weight

Variability for mean values of 19 quantitative traits in nine groups identified by Manhattan dissimilarity coefficient

1   Table 4.Variability for mean values of 19 quantitative traits in nine groups identified by Manhattan dissimilarity coefficient

Trait	Number of genotypes in each cluster								
	7	25	4	4	3	2	1	1	1
Days to 50% flowering	72.86	50.24	50.50	44.88	57.67	62.00	53.50	56.50	70.50
Plant height (cm)	126.56	152.87	174.28	139.36	172.68	139.78	125.45	135.95	151.25
Head Diameter (cm)	1.66	1.55	1.49	1.59	1.41	1.16	1.65	1.45	1.03
Panicle length (cm)	23.71	23.68	25.99	23.90	30.53	19.23	22.00	26.00	17.95
Productive tillers per plant	3.67	4.98	4.36	5.29	4.70	6.70	5.90	4.70	3.50
Grain yield per plant	24.72	29.63	48.99	22.53	27.23	22.33	47.75	15.85	28.35
Days to maturity	86.50	85.12	86.13	88.38	87.00	81.50	85.00	86.00	84.00
1000 grain weight	7.83	7.12	7.79	6.50	7.04	6.32	7.27	5.71	6.16
Protein content (%)	12.50	14.49	12.72	12.61	9.71	13.69	20.06	15.72	15.71
Lipid content (%)	5.67	4.36	4.72	5.64	3.25	4.13	2.82	6.92	6.64
Iron content (ppm)	52.03	50.39	37.14	55.66	46.22	43.33	69.65	48.98	46.16
Zinc content (ppm)	36.59	40.25	35.21	47.05	36.07	30.54	35.13	44.98	48.79
Manganese content (ppm)	13.63	14.94	13.65	13.30	14.40	9.66	17.28	7.20	10.39
Calcium content (ppm)	191.57	208.04	144.75	207.25	215.83	158.75	229.00	204.50	218.50
Copper content (ppm)	9.40	8.02	7.19	13.85	19.78	9.44	8.18	17.29	19.29
Potassium content (ppm)	45.70	49.33	46.08	49.31	49.00	45.58	52.30	18.00	60.15
Phosphorus content (ppm)	31.43	31.81	30.68	27.54	31.98	29.23	27.75	31.52	32.07
Phytate content (mg/100g)	283.74	272.46	327.68	248.10	398.32	206.55	287.75	307.10	250.95
Total phenolic acid (mg/100g)	58.59	58.50	58.21	66.53	65.50	59.57	75.16	56.96	73.15

Amplification details of DNA markers

1   Table 5. Amplification details of DNA markers

SSR marker system

Maker name	Band size (bp)	TB	Polymorphism (%)	PI	PIC	RP	Mean RP
PMES153	145-154	4	100	1.14	0.29	2.00	0.50
PMES157	135-151	4	100	1.46	0.37	1.96	0.49
PMES160	134-150	4	100	1.36	0.34	2.13	0.53
PMES161	155-233	3	100	1.23	0.41	2.04	0.68
PMES162	132-162	7	100	1.35	0.19	1.63	0.23
PMES168	222-284	10	100	1.96	0.19	2.38	0.24
PMES170	154-195	9	100	1.43	0.16	2.00	0.22
PMES171	151-292	13	100	1.71	0.13	2.00	0.15
PMES173	216-203	8	100	0.99	0.50	2.00	1.00
PMES185	176-200	8	100	1.55	0.19	1.83	0.23
PMES190	85-104	6	100	1.53	0.26	1.92	0.32
PMES199	213-305	4	100	1.27	0.32	1.83	0.46
Average		6.17		1.42	0.28	1.98	0.42
SRAP marker system							
Em6+Me2	205-1234	25	100	8.11	0.32	13.25	0.53
Em2+Me2	94-1152	34	100	7.96	0.26	10.20	0.30
Em1+Me2	223-1125	18	100	5.33	0.30	6.88	0.38
Em5+Me4	120-1020	17	100	3.81	0.22	4.63	0.27
Em6+Me3	450-1357	10	100	2.33	0.23	5.42	0.54
Em2+Me3	313-1065	26	100	6.57	0.25	9.25	0.36
Average		21.67		5.69	0.26	8.27	0.4

2   TB: Total Number of Bands; PI: Primer index; PIC: Polymorphic Information Content; RP: Resolving
3   Power.

Comparison between SSR and SRAP marker system

1 Table 6. Comparison between SSR and SRAP marker system

Marker system	Total markers	TB	PB	FP	H_{av}	MR	EMR	MI
SSR	12	74.00	74.00	1.00	3.35	5.69	5.69	19.092
SRAP	6	130.00	130.00	1.00	1.57	10.00	10.00	15.655

2 TB: Total Number of Bands; PB: Number Polymorphic Bands; PI: Primer index; PIC:
3 PolymorphicInformation Content; RP: Resolving Power; FP: Fractionation of Polymorphism; Hav:
4 Average PIC; MR: Multiplex Ratio; EMR: Effective Multiplex Ratio; MI: Marker Index.

Figure 1

UPGMA based dendrogram with Manhattan dissimilarity coefficient of studied traits
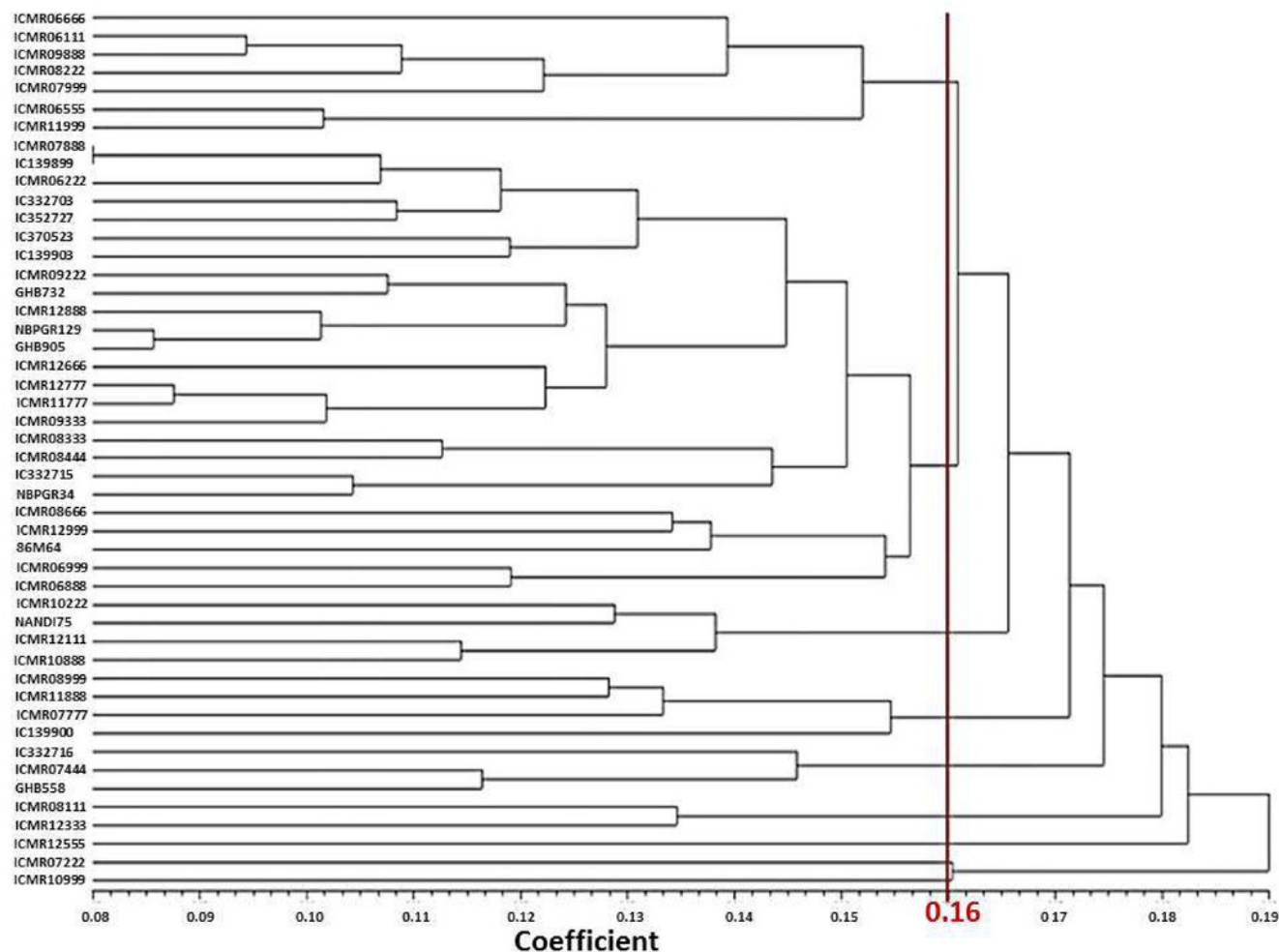
Figure 2

UPGMA based dendrogram with Jaccard's similarity coefficient of DNA markers