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

Darshanaben F. Gunguniya[1], Sushil Kumar[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, 388 110, India

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

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

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

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

Abstract

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.

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

1. Introduction

Pearl millet [*Pennisetum glaucum*(L.)] is a small-seeded C4 plant of the Poaceae family. The 1.7 GB genome of this crop is accommodated by 2n=2x=14 chromosomes. Compared to other cereals like wheat and rice, it can withstand effectively in drought, nutrient-depleted soil, and hot environmental conditions of the hostile deserts of India and Africa. This hardy nature makes pearl millet resilient to harsher climatic conditions consequently it is cultivated in marginal environments of arid and semi-arid tropical regions of India and south east Asia, sub-Saharan Africa and much of southern and eastern Africa (Ramya et al., 2018). Pearl millet is a key food for millions living the semi-arid and arid regions. The grain is mainly consumed as human food while biological yield is used as livestock feed. ~~pearl~~Pearl millet is a primary food for mankind living in dryland agriculture regions.

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

In any breeding strategy, variation continues to be the key to success. Pearl millet shows abundant phenotypic variability for most of the quantitative traits like flowering time, ear head length, grain characteristics, tolerance to various (a)biotic stresses as well as nutritional quality (Bhattacharjee et al., 2007). Effective and logical utilization of this diversity is vital to any breeding program (Allard et al., 1960). Exploiting this genetic diversity in the pearl millet 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. In comparison to wheat (11.8 g/100 g), the protein in this coarse cereal 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 been employed for genetic diversity assessment (Li and Quiros, 2001). The current study was created to analyse the natural variability for grain mineral and anti-nutrient content in grain as

Açıklama [H1]: This is incorrect information and you haven't fixed it despite R2 warning. Please, correct it. Give a range instead of a single value. This value ranged from about 7% to 22%

Biçimlendirilmiş: Vurgulu

well as molecular diversity in pearl millet germplasm with the objective of improving the nutritional quality and food safety of pearl millet as well as expanding understanding in this area.

Materials and methods

The field trial was done in a randomized complete block design (RCBD) with two replications. The inter- and intra-row distance was 60 and 15 cm, respectively. The recommendations for crop management practices were followed for uniform plant growth and a healthy crop stand. The seeds were sown on February 2021. A total of 48 pearl millet genotypes were used for the study (Supp Table 1).

1.1. Morphological characters

The panicles were covered with glassine bags to prevent cross-pollination by outside pollen and to collect self-seeds. For phenotyping of grain based traits, physiologically mature panicles were collected, dried under sunlight, and then manually threshed in bulk.

The experimental material was evaluated for eight morphological traits viz., days to 50% flowering, plant height, panicle diameter (PD, cm), panicle length (PL, cm), number of productive tillers, grain yield, days to maturity, and 1000 grain weight. Except for days to 50% flowering and days to maturity, which were recorded on a plot basis during the study, data on the above traits were collected from randomly tagged five competitive plants in each genotype in both replications. PD was measured with Vernier calipers.

1.2. Biochemical characters

Before biochemical analysis, grains were cleaned followed by hot air oven drying (80°C for 24 h). Dried grains (10 g) were powdered manually. For mineral analysis, 0.5 g flour was processed in 11 mL of nitric acid (69%) and 1 mL of H_2O_2. The digestions were carried out in HVT50 vessels using rotor 12HVT50 in Multiwave GO/ Multiwave GO plus (Anton Paar GmbH, Austria). For microwave digestion the initial temperature was kept at 180°C with a ramp of 20 minutes and a hold period of 12 minutes. While the second round of digestion was performed with a ramp time of 10 mins at 70°C temperature and a holding time of 5 mins. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES (model 7000DV of make: Perkin Elmer, made in USA with wintab32 software ver. 5.1) was used to determine the mineral

content (Fe, Zn, Ca, Cu, and Mn) in grain after diluting the digested mixture to a volume of 50 ml using distilled water. The flow rate in a peristaltic pump was 1.5ml / min. From the acid extract, potassium content was quantified using a flame photometer (Jackson, 1973), while the vanadate-molybdate method of Jackson (1973) was used to estimate the phosphorus. Total phenols were estimated using the Folin-Ciocalteau reagent as Malik and Singh (1980) and reading was measured at 730 nm using a spectrophotometer. Soxhlet extraction was performed to estimate the crude oil content, and semi micro-Kjeldahl was employed to determine the crude protein content.

1.3. Molecular marker study

Genomic DNA was extracted from tender leaves as per Mace et al (2003). Genotyping was done using simple sequence repeat (SSR) and sequence-related amplified polymorphism (SRAP) markers. For SSR marker profiling, markers from the PMES series (Zala et al., 2017) were amplified in SensoQuest Thermocycler (Germany). The SSR-PCR reaction conditions were as follows: 94°C (initial denaturation) for 5 min., followed by 35 cycles of 94°C for 45 sec, X°C (primer specific) for 45 sec, 72°C for 45sec, and 72°C for 7 min (final extension) . The SRAP amplification was as follows: 94 °C (initial denaturation) for 5 min., followed by 5 cycles 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 (primer specific) for 45 s, 72 °C for 60 s and 72 °C for 10 min (final extension). An agarose gel (3%) was used to resolve PCR products.

1.4. Statistical analysis

The mean value of traits was figured out, and analysis of variance (ANOVA) was performed in accordance with Panse and Sukhatme (1978) in Microsoft Excel 2013. A phenotypic trait-based dissimilarity matrix was constructed using Manhattan coefficients with Numerical Taxonomy and Multivariate Analysis System (NTSYSpc 2.0; Rohlf 1998). The amplified products of SSR and SRAP markers were scored in 1 (presence) and 0 (absence) fashion. Polymorphism information content (PIC), Multiplex ratio (MR), effective multiplex ratio (EMR) marker index (MI) and resolving power (Rp) value were estimated following Sharma et al. (2016) in Microsoft Excel 2013. In NTSYSpc 2.0, The SIMQUAL program used Jaccard's similarity (J) coefficient to compute the genetic similarity between genotypes, SAHN clustering method was used to

construct the unweighted pair group method with arithmetic mean (UPGMA) dendrogram (Sneath and Sokal, 1973).

3. Results and discussion

The ANOVA resulted that genotypic variations were significant at a 5% level of probability for all the traits, showing ample genetic diversity among the genotypes under study (Table 1). This also suggested that there is sufficient scope to select superior breeding material which can be exploited in pearl millet breeding programs.

3.1. Character variance analysis

3.1.1. Morphological parameters

Early flowering is a desirable trait for pearl millet as it is a crop of semi-arid and arid regions. Earliness becomes an important trait in areas where scanty and erratic rains aggravate the moisture stress condition during the growth stage of the crop and leads to post-flowering moisture stress (Yadav et al., 2011). In the current study, though, the population mean for days to 50% flowering was 54.69 days but the days to 50% flowering ranged from 34 (IC 370523) to 77 days (ICMR 07999). Earlier literature also recorded similar values for days to 50% flowering for example 49.06 days by Govindaraj et al. (2011), 53.10 days by Sonali et al. (2019) and 55.61 days by Pallavi et al. (2020). PH is an important trait that governs tradeoffs between competition and resource distribution, which is decisive for productivity (He et al., 2021). Semi-dwarf genotypes are better than their tall counterparts because of their reduced lodging vulnerability and better response to nitrogen Azhaguvel et al. (2003). In the present experiment, PH ranged from 110.10 cm (ICMR 06555) to 205.35 cm (IC 332715) with an average of 149.42 cm. The results indicated that most of the studied genotypes are semi-dwarf in nature and with better management dwarfism supports the grain yield.

Panicle size (length and diameter) are two important traits that have direct positive correlations with grain yield in pearl millet (Vengadessan et al., 2013). Hence, the improvement of sink-size linked traits is a key objective for pearl millet improvement programs. PL in the present study ranged from 15.55 cm (ICMR 11888) to 38.05 cm (IC-332716) with an average of 24.03 while the diameter ranged from 1.03 cm (ICMR 10999) to 2.15 cm (ICMR 09333) with an average of

1.53 cm. Abubakar et al. (2019) observed a similar range and mean in pearl millet (2.26 cm) for panicle diameter. Similarly, results for PL are comparable with previous reports (Sharma et al., 2018, Rani et al., 2019). The number of productive tillers per plant varied from 2.65 (ICMR 08222, ICMR 11999) to 7.60 (IC 370523) with an average of 4.80 (Table 2). According to Sile et al. (2004), non-tillering millet genotypes produced bold seeds having TGW >10gm than the genotype that produced tillers. Similarly, Maman et al. (2004) also reported that, a reduction in productive tillers from 10 to 3 or 5 improved seed yields by 15-30%. Yadav et al (2021) reported that private-sector hybrids are generally ~~having~~ have less effective tillers/plant. But still, farmers in drought-prone areas prefer high tillering hybrids because tillering is a strategy of adaptation to intermittent drought spells (Yadav et al., 2016).

In cereal breeding, yield, a complex trait, is one of the supreme traits which is influenced by several associated traits. 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. Large variability was also observed for1000 grain weight (TGW) which is determined by the form, size and density of the grain and these are directly related to total grain yield. TGW ranged from 4.93 g (ICMR 06888) to 10.45 g (ICMR 06555) with an average of 7.14 g. A diversity assessment of 21,594 pearl millet genotypes from 50 nations revealed huge variability for the TGW (1.5 to 21.3 g) (Upadhyaya et al., 2007). Three-fold variability for TGW (6-16 g) was earlier recorded by Pujar et al (2018).

3.1.2. Biochemical parameters

Compared to other main cereal crops, pearl millet yields more nutritious grains with protein, calcium, phosphorus, iron, and zinc (Devos et al., 2006). Currently, the commercially grown varieties/hybrids of pearl millet produce grains with an average Fe and Zn content of 42 and 32 ppm (parts per million), respectively (Rai et al. 2016). However, a much wider variability for these micro-nutrients has been reported in germplasm collections (Rai et al. 2014). Iron is an essential element for blood production and for the growth and development of the body. Zinc is essential for the development of a strong immune system. The values of iron content in the current study ranged from 31.58 (ICMR 07777) to 77.38 (ICMR 08666) with an average of 49.69. Zinc content ranged from 29.34 (ICMR 08111) to 55.48 (IC 139903) with an average of 39.36 (Table 2). In previous studies, values ranging from 45.50-55.73 for grain Fe concentration and between 38.60-46.61 for grain Zn concentration have been reported (~~A similar mean value~~

was observed by (Velu et al., (2007;) where grain Fe was 45.50 ppm. In other studies grain Fe and Zn was varied with change of germplasm for example it was 57.65 ppm of Fe and 46.61 ppm of Zn in Anuradha et al. (2017; 2018;);, 55.73 ppm of Fe and 42.75 ppm of Zn in Anuradha et al. (2018); 53.57 ppm of Fe) and 40.39 ppm of Zn in Sonali et al. (2019); and; 50.60 ppm of Fe) and 38.60 ppm of Zn in Yadav et al. (2020).

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

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

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

The transport of water, nutrients, and carbohydrates within plant cells is linked with potassium. It is a crucial mineral for the activation of several enzymes that control the synthesis of protein, starch, and adenosine triphosphate (ATP) in plants. The potassium ranged from 1800 ppm (ICMR 07222) to 6000 ppm (ICMR 10999) with an average of 4700 ppm. Large variability for

potassium was also recorded in 122 commercial pearl millet cultivars (3675–5375ppm; Govindaraj et al. 2020a) and core collection (3667-5133 ppm; Govindaraj et al. 2020b).

The body needs phosphorus to produce protein for the development, upkeep, and repair of cells and tissues. Additionally, it participates in the production of ATP. The values of phosphorus content ranged from 2200 (IC 139900) to 3600 ppm (ICMR 08666) with an average of 3112 ppm. ICMR 06555 was statistically at par with IC 139900.

Pearl millet is also a promising source of protein. Studies indicated that protein in pearl millet is circa 11.8 %, which is better than rice (8.6 %), and maize (9.2 %) and comparable with sorghum (10.7 %). Moreover, pearl millet grain is enriched with glutamate which is a precursor of γ-aminobutyric acid (GABA) (Tomar et al., 2021). In the current study the protein content ranged from 8.26% (IC 332716) to 20.06% (ICMR 12555) with an average of 13.73%. ICMR 07444 (9.89%) was statistically at par with IC 332716. The study of Pujar et al. (2020) reported grain protein content variation between 6 - 18%, with a mean of 11%. The augmentation of pearl millet in daily food can reduce the risk of protein malnutrition in an economical way. Moreover, protein extracted from pearl millet can be exploited to design protein-enriched functional foods.

The lipid content ranged from 2.72% (ICMR 06999) to 6.95% (ICMR 08444) with an average of 4.68%. A comparable range and mean were observed by Arulselvi et al. (2007; 5.12%), Abdalla et al. (2007; 2.70-7.10%) and Tomar et al. (2021; 5.24-9.99). The lipid content in pearl millet ranges from 1.5 to 6.8% which is higher sorghum and other millets (Hassan et al., 2021). Though, the high lipids have been documented as possible causes for the rancidity of millet flour. However, the shelf life of flour can be increased by hydrothermal treatment, irradiation, cooling storage, or a combination of more than one technology (Goyal and Chugh, 2017).

The metal-chelating ability of phytic acid makes it is an antinutritional phytochemical as it declines the bio-availability of ions like Mn, Ca, Mg, Fe and Zn (Marathe et al., 2018). In the current study, the phytate ranged from 201.5 mg/100g (ICMR 08111) to 542.50 mg/100g (GHB 558) with an average of 282.39. Abdalla et al. (2007) also recorded a similar range from 354-795 mg/100g of phytate. ~~where ;). The study by~~ Gabaza et al. (2018) reported that phytate in pearl millet grains ranges between 580 mg/100g to 1380 mg/100g which is similar to sorghum and maize. The range of phytate in the current study is supported by the result of Pushparaj and

Urooj (2014) in Indian cultivars where it was between 0.26 - 0.99 g/100 g. The result suggested that phytic acid content in pearl millet grain is significantly lower than in rice (0.68-1.03%; Liu, 2005), oat (0.5–1.2%; Peterson, 2001), soybean (1.0–2.22%; Lolas et al. 1976) and wheat (0.2 - 2.9%; Gupta et al. 2015).Hence, regular consumption will possibly not hamper the bioavailability of minerals.

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

3.2. Nutrient-dense genotypes

Genotypes dense in multiple nutrients can directly be released as a variety after evaluating their yield performance over the locations for multiple years. Such genotypes can be exploited in a hybridization program. In the current study, the top eight nutrient-dense genotypes flowered in 34–74 days and had 5.71–9.39 g TGW (Table 3). Top genotypes had Fe content of 61.07-77.38 (ICMR 08666) ppm, Zn content of 45.11-55.48 (IC 139900) ppm, Mn content of 16.2-17.63 (ICMR 08444) ppm, Ca content of 230.5-256 (ICMR 12888) ppm, Cu content of14.32-22.59 (GHB 558) ppm, K content of52.3-60.15(ICMR 10999) ppm, and P content of 33.28-36.72 (ICMR 08666). IC 139900 was superior for both Fe (71.22 ppm) and Zn (55.48 ppm). Genotype ICMR 08666 was dense for Fe, Zn, K, and P. Out of eight high-Fe genotypes, only two genotypes had > 75 ppm. Thus current experiment also identified the best genotypes that had a higher content of multiple nutrients. Earlier, Govindraj et al (2020) also identified genotypes having a high content of multiple nutrients.

3.3. Phenotypic diversity analysis

Phenotypic diversity is important for pearl millet breeding. The interactions between the genome and all of its growing micro- and mega-environments lead to the phenotype of the plant (Fasoula et al., 2020). The mean value of each trait was used to generate the Manhattan dissimilarity coefficient and dendrogram (Sokal and Michener, 1958). The genotypes were divided into nine

major clusters based on the Manhattan dissimilarity coefficient. Earlier, Shashibhushan et al (2022) also generated eight clusters of 40 pearl millet genotypes using phenotypic data. In current study, the average dissimilarity value among genotypes was calculated to be 0.16, demonstrating modest phenotypic variability (Table 4). The dissimilarity between genotypes ranged from 0.08 (IC 139899 and ICMR 07888) to 0.27 (Nandi 75 and ICMR 07222) for the respective pair of genotypes.

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

3.4.Molecular marker based diversity

Forty-eight genotypes of pearl millet were analyzed using SSRs and SRAP markers (Table 5). During the experiment, a total of 50 SSR markers were screened for amplification. Out of 50 markers, 32 (60%) primers showed amplification. Of these 30 SSR markers, 12 (37.5%) markers were found polymorphic. These 12 polymorphic SSRs markers generated 65 amplicons. The molecular weight of the amplicon ranged from 85bp (PMES 190) to 292bp (PMES 171). In previous reports with PMES-series SSR markers, Zala et al. (2017) recorded amplicon size from 101 to 285 bp. The number of polymorphic bands/amplicons per SSR marker ranged from 2 to 13, with a mean of 6.71. All SSR amplicons were found polymorphic. The PIC value, the informativeness of a primer, for each marker was computed for the estimation of marker allelic variation considering the allele frequencies in studied genotypes. The mean PIC of SSR markers was 0.28 though it varied from 0.132 (PMES 171) to 0.499 (PMES 173). This range was comparable with Zala et al. (2017) where PIC was between 0.188 - 0.375.

Açıklama [H2]: Is this 32 or 30? Which one?

The Rp was estimated considering the proportion of genotypes containing the amplicon. The primer that might best differentiate the cultivar can easily be identified by the value of the Rp and PIC. In the current study, Rp varied from 1.625 (PMES 162) to 2.375 (PMES 168), with an average of 1.98. Mean Rp was between 0.154 (PMES 171) to 1.000 (PMES 173). The PI ranged from 0.998 (PMES 173) to 1.963 (PMES 168), though the mean PI value was 1.420. MI is considered to be an inclusive measure of the efficiency to detect polymorphism. The SSR MI was 19.092.

In the case of the SRAP markers, of 25 SRAP, 6 (24%) were polymorphic. The polymorphic SRAPs amplified 119 amplicons. The product size for SRAPs ranged from 94 (Em2+Me2) to 1357 bp (Em6+Me3). The polymorphic bands ranged from 10 (Em6+Me3) to 34 (Em2+Me2), with a mean of 21.67. Liu et al. (2008) observed a polymorphic band detected with each ranging from 6 to 17, with an average of 11.76. Bhatt et al. (2017) had a band size from 120 to 500 bp in cumin. This suggested that in different crops SRAP amplicon size will be highly variable. PIC oscillated from 0.224 (Em5+Me4) - 0.324 (Em6+Me2), with an average of 0.26. Bhatt et al. (2017) reported a PIC value (0.34).

The mean PI of SRAP markers was 5.69, through the maximum PI was for Em6+Me2 (8.108) and the lowest value for Em6+Me3 (2.335). Rp ranged from 4.625 (Em5+Me4) to 13.250 (Em6+Me2), with an average value of 8.27. Mean Rp was between 0.272 (Em5+Me4) - 0.542 (Em6+Me3) with an average of 0.40. Liu et al. (2008) stated higher RP values ranged from 2.229 to 8.457 with an average of 4.927. The fraction of polymorphism, MR, EMR and MI for SRAPs are 1.00, 10.00, 10.00, and 15.65, respectively (Table 6).

3.5. Inter-Genotype Genetic Relationship

Forty-eight pearl millet genotypes were divided into seven major groups by the dendrogram created using pooled data from SSR and SRAP markers based on Jaccard's similarity matrix. Cluster I, II, III, IV, V, VI and VII had 1, 1, 11, 8, 3, 22, and 2 genotypes, respectively (Figure 1). In the study of Nehra et al. (2017), with SSR markers, 49 accessions were clustered into eight core clusters. Kumar et al. (2020b) alienated 18 lines into three clusters in pearl millet using 74 SSRs. In the current study, the inter-accession genetic coefficient of similarity ranged from 0.616 to 0.877 while the average similarity was 0.743. ICMR 098888 and GHB 905 has a genetic

distance (0.384) indicative that both genotypes are having moderate genetic ~~genomic~~ difference level and can be crossed to create a bi-parental mapping population. The minimum genetic distance (0.123) was between IC 139899 and IC 332727, demonstrating that these accessions have more similarity in ~~microsatellite~~ SSR locus. Moreover, based on diversity results, breeders can select diverse genotypes for combining ability and heterosis analysis for traits studied in the current study.

4. Conclusion

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

5. Conflict of Interest

All authors declared that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

6. Authors' Contribution

SK, MPP, AAS & VK: Conceptualization & Designing the experiment, **DFG, RP & DK**: performed the experiments, **RP & SK**: analyzing the data, **VK:** Contribution of experimental material, **DFG, SK & RP**: Prepared the figures and tables, **SK & AAS**: Critically revised the manuscript for important content,

7. Funding

There are no funding sources.

8. Acknowledgments

All authors would like to thankful to Anand Agricultural University for providing necessary facilities and resources.

References

Abubakar, A., Falusi, O. A., Adebola, M. O., Olayemi, I. K., & Daudu, O. A. Y. (2019). Genetic diversity studies for morphological traits in pearl millet (*Pennisetum glaucum* L.) landraces of Northern Nigeria. GSC Biological and Pharmaceutical Sciences, 7(2), 60–70

Allard, R. W. (1960). *Principles of Plant Breeding*. New York: John Willey and Sons. Inc

Anuradha, N., Satyavathi, C. T., Bharadwaj, C., Nepolean, T., Sankar, S. M., Singh, S. P., & Srivastava, R. K. (2017). Deciphering genomic regions for high grain iron and zinc content using association mapping in pearl millet. *Frontiers in Plant Science*, 8, 412.

Anuradha, N., Satyavathi, C. T., Bharadwaj, C., Sankar, M., & Pathy, L. (2018). Association of agronomic traits and micronutrients in pearl millet. *International Journal of Chemistry Studies*, 6, 181-184.

Anuradha, N., Satyavathi, C. T., Bharadwaj, C., Sankar, M., Singh, S. P. and Pathy T. L. (2018). Pearl millet genetic variability for grain yield and micronutrients in the arid zone of India. Journal of Pharmacognosy and Phytochemistry 7(1): 875-878

Anuradha, N., Satyavathi, C. T., Meena, M. C., Sankar, S. M., Bharadwaj, C., Bhat, J., & Singh, S. P. (2017). Evaluation of pearl millet [*Pennisetum glaucum* (L.) R. Br.] for grain iron and zinc content in different agro climatic zones of India. *Indian Journal of Genetics and Plant Breeding*, 77(1), 65-73.

Arora, P., Sehgal, S., & Kawatra, A. (2003). Content and HCl-extractability of minerals as affected by acid treatment of pearl millet. *Food Chemistry*, 80(1), 141-144.

Arulselvi, S., Mohanasundaram, K., Selvi, B., & Malarvizhi, P. (2007). Genetic variability studies and interrelationships among nutritional quality characters, phytate phosphorus and grain yield in the seeds of pearl millet *Pennisetum glucum* (L.) R. Br. *Indian Journal of Genetics and Plant Breeding*, 67(1), 37-40.

Azhaguvel, P., Hash, C. T., Rangasamy, P., & Sharma, A. (2003). Mapping the d 1 and d 2 dwarfing genes and the purple foliage color locus P in pearl millet. *Journal of Heredity*, 94(2), 155-159.

Bhatt, J., Kumar, S., Patel, S., & Solanki, R. (2017). Sequence-related amplified polymorphism (SRAP) markers based genetic diversity analysis of cumin genotypes. *Annals of Agrarian Science*, 15(4), 434-438.

Bhattacharjee, R., Khairwal, I. S., Bramel, P. J., & Reddy, K. N. (2007) Establishment of a pearl millet [*Pennisetum glaucum* (L.) R. Br.] core collection based on geographical distribution and quantitative traits. *Euphytica*, 155:35-45.

Devos, K. M., Hanna, W. W. and Ozias-Akins, P. (2006). Pearl millet. In: C. Kole (eds): Genome Mapping and Molecular Breeding in Plants, Volume 1, Cereals and Millets. Springer–Verlag Berlin Heidelberg

Fasoula, D. A., Ioannides, I. M., & Omirou, M. (2020). Phenotyping and plant breeding: overcoming the barriers. *Frontiers in plant science*, *10*, 1713.

Hassan, Z. M., Sebola, N. A. & Mabelebele, M. (2021). The nutritional use of millet grain for food and feed: a review. Agric Food Secur. 10(1): 16

Gabaza, M., Shumoy, H., Muchuweti, M., Vandamme, P., & Raes, K. (2018). Baobab fruit pulp and mopane worm as potential functional ingredients to improve the iron and zinc content and bio accessibility of fermented cereals. *Innovative Food Science & Emerging Technologies*, 47, 390-398.

Glaszmann, J. C., Kilian, B., Upadhyaya, H. D., & Varshney, R. K. (2010). Accessing genetic diversity for crop improvement. *Current opinion in plant biology*, *13*(2), 167-173.

Gopalan, C., Rama Sastri, B. V., & Balasubramanian, S. C. (2003). *Nutritive Value of Indian Foods*. 156.

Govindaraj, M., Rai, K. N., Kanatti, A., Upadhyaya, H. D., Shivade, H., & Rao, A. S. (2020). Exploring the genetic variability and diversity of pearl millet core collection germplasm for grain nutritional traits improvement. *Scientific Reports*, 10(1), 1-13.

Govindaraj, M., Selvi, B., Rajarathinam, S., & Sumathi, P. (2011). Genetic variability and heritability of grain yield components and grain mineral concentration in India's pearl millet (*Pennisetum glaucum* (L) R. Br.) accessions. *African Journal of Food, Agriculture, Nutrition and Development*, 11(3), 4758-4771.

Goyal, P., & Chugh, L. K. (2017). Shelf-life determinants and enzyme activities of pearl millet: a comparison of changes in stored flour of hybrids, CMS lines, inbreds and composites. *Journal of food science and technology*, 54(10), 3161-3169.

Gupta RK, Gangoliya SS and Singh NK. (2015) Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. J Food Sci Technol. 52(2), 676-84

He, Q., Zhi, H., Tang, S. Xing, L., Wang, S., Wang, H., Zhang, A., Li, Y., Gao, M., Zhang, H., Chen, G., Dai, S., Li, J., Yang, J., Liu, H., Zhang, W., Jia, Y., Li, S., Liu, J., Qiao, Z.. (2021). QTL mapping for foxtail millet plant height in multi-environment using an ultra-high density bin map. *Theoretical and Applied Genetics*, 134, 557–572.

Jackson, M. L. (1973). Soil chemical analysis, pentice hall of India Pvt. *Ltd., New Delhi, India*, *498*, 151-154.

Kaur, K. D., Jha, A., Sabikhi, L., & Singh, A. K. (2014). Significance of coarse cereals in health and nutrition: a review. *Journal of Food Science and Technology*, 51(8), 1429-1441.

Kaushik, I. & Grewal, R. B. (2017). Antinutrient and mineral content of thirteen different varieties of pearl millet locally grown in Haryana, India. *International Journal of Current Microbiology and Applied Sciences,* 6(5): 2136-2143.

Kumar, M., Rani, K., Ajay, B. C., Patel, M. S., Mungra, K. D., & Patel, M. P. (2020a). Multivariate diversity analysis for grain micronutrients concentration, yield and agro-

morphological traits in Pearl millet (*Pennisetum glaucum* (L) R. Br.). *International Journal of Current Microbiology and Applied Sciences*, 9(3), 2209-2226.

Kumar, S., Hash, C. T., Thirunavukkarasu, N., Singh, G., Rajaram, V., Rathore, A., & Srivastava, R. K. (2016). Mapping quantitative trait loci controlling high iron and zinc content in self and open pollinated grains of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. *Frontiers in Plant Science*, 7, 1636.

Kumar, S., Hash C.T., Singh, G., Basava, R.K. & Srivastava, R.K. (2020b) Identification of polymorphic SSR markers in elite genotypes of pearl millet and diversity analysis, *Ecological Genetics and Genomics*, 14, 100051

Li, G., & Quiros, C. F. (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. *Theoretical and Applied Genetics*, 103(2), 455-461.

Liu Z (2005) Grain phytic acid content in japonica rice as affected by cultivar and environment and its relation to protein content. Food Chem 89:49–52

Liu, L. W., Zhao, L. P., Gong, Y. Q., Wang, M. X., Chen, L. M., Yang, J. L., & Wang, L. Z. (2008). DNA fingerprinting and genetic diversity analysis of late-bolting radish cultivars with RAPD, ISSR and SRAP markers. *Scientia Horticulturae*, 116(3), 240-247.

Lolas GM, Palamidids N and Markakis P. (1976)_The phytic acid—total phosphorus relationship in barley, oats, soybeans and wheat. Cereal Chem. 53, 867–871

Mace, E. S., Buhariwalla, K. K., Buhariwalla, H. K., & Crouch, J. H. (2003). A high-throughput DNA extraction protocol for tropical molecular breeding programs. *Plant molecular biology reporter*, *21*(4), 459-460.

Malik, C. P. and Singh, M. B . (1980). Plant enzymology and histo-enzymology. Food and Agricultural Organization of the United Nations 286.

Maman, N., Mason, S. C., Lyon, D. J., and Dhungana, P. (2004). Yield components of pearl millet and grain sorghum across environments in the Central Great Plains. *Crop Science*, *44*(6), 2138-2145.

Maman, N., Mason, S., Lyon, D. J. and Dhungana, P. (2004). Yield components of pearlmillet and grain sorghum across environments in the central great plains. *Panhandle Research and Extension Centre*. Paper 3.

Marathe, A., Krishnan, V., Vinutha, T., Dahuja, A., Jolly, M., & Sachdev, A. (2018). Exploring the role of Inositol 1, 3, 4-trisphosphate 5/6 kinase-2 (GmITPK2) as a dehydration and salinity stress regulator in Glycine max (L.) Merr. through heterologous expression in E. coli. *Plant Physiology and Biochemistry*, *123*, 331-341.

Nehra, M., Kumar, M., Vart, D., Kaushik, J., & Sharma, R. K. (2017). Molecular characterization of pearl millet [*Pennisetum glaucum* (L.) R. Br] inbreds using microsatellite markers. *Journal of Applied and Natural Science*, *9*(1), 357-363.

Oshodi, A. A., Oqungbenle, H. N. and Oladimeji, M. O. (1999). Chemical composition, nutritionally valuable minerals and functional properties of benniseed (*Sesamum radiatum*), pearl millet (*Pennisetum typoides*) and quinoa (*Chenopodium quinoa*) flours. *Int. J. Food Sci. Nutr*., 50: 325-331.

Pallavi, M., Reddy, P. S., Krishna, K. R., Ratnavathi, C. V., & Sujatha, P. (2020). Genetic variability, heritability and association of grain yield characters in pearl millet (*Pennisetum glaucum* L). *Journal of Pharmacognosy and Phytochemistry*, 9(3), 1666-1669.

Panse, V. G., & Sukhatme, P. V. (1978). Statistical Methods for Agricultural Worker. Indian Council of Agricultural. Research, New Delhi, India

Peterson D.M. (2001) Oat antioxidants J. Cereal Sci., 33,. 115-129

Pedraza-Garcia F, Specht JE, Dweikat I (2010) A new PCR-based linkage map in pearl millet.Crop Sci 50:1754–1760

512 Pujar, M., Gangaprasad, S., Govindaraj, M. and Kanatti, A. (2018).Genetic diversity analysis
513 among advanced breeding lines in pearl millet for grain iron, zinc and agronomic traits.
514 *In*: 1st National Genetics Congress on Genetics for Sustainable Food, Health and
515 Nutrition Security, December 14-16, 2018, ICAR-lndian Agricultural Research
516 Institute, New Delhi, India.

517 Pujar, M., Govindaraj, M., Gangaprasad, S., Kanatti, A., & Shivade, H. (2020). Genetic variation
518 and diversity for grain iron, zinc, protein and agronomic traits in advanced breeding
519 lines of pearl millet [*Pennisetum glaucum* (L.) R. Br.] for biofortification breeding.
520 *Genetic Resources and Crop Evolution*, 67, 2009-2022.

521 Pushparaj, F. S., & Urooj, A. (2014). Antioxidant activity in two pearl millet (*Pennisetum*
522 *typhoideum*) cultivars as influenced by processing. *Antioxidants*, 3, 55-66.

523 Rai, K. N., Gupta, S. K., Sharma, R., Govindaraj, M., Rao, A. S., Shivade, H., & Bonamigo, L.
524 A. (2014). Pearl millet breeding lines developed at ICRISAT: a reservoir of variability
525 and useful source of non-target traits. *SAT eJournal*, *1*(1), 1-13.

526 Rai, K. N., Yadav, O. P., Govindaraj, M., Pfeiffer, W. H., Yadav, H. P., Rajpurohit, B. S., &
527 Shivade, H. (2016). Grain iron and zinc densities in released and commercial cultivars
528 of pearl millet (Pennisetum glaucum). *Indian Journal of Agricultural Sciences*, *86*(03),
529 11-16.

530 Ramya, A. R., Ahamed M, L., Satyavathi, C. T., Rathore, A., Katiyar, P., Raj, A. G. B., Kumar,
531 S., Gupta, R., Mahendrakar, M.D., Yadav, R.S. & Srivastava, R. K. (2018). Towards
532 defining heterotic gene pools in pearl millet [*Pennisetum glaucum* (L.) R. Br.].
533 *Frontiers in Plant Science*, 8, 1934.

534 Rani S., T., Sameer Kumar, C. V., Maheswaramma, S., Parimal, M., Kumar, A., & Sravanthi, K.
535 (2019). Selection criteria for grain yield in pearl millet (*Pennisetum glaucam* L.) in
536 association with yield contributing traits. *International Journal of Pure & Applied*
537 *Bioscience*, *7*(3), 257-262.

538 Rohlf, F. J. (1998). NTSYS-pc: Microcomputer programs for numerical taxonomy and
539 multivariate analysis. *The American Statistician*, 41(4), 330-330.

Sharma, B., Chugh, L. K., Sheoran, R. K., Singh, V. K., & Sood, M. (2018). Study on genetic variability, heritability and correlation in pearl millets germplasm. *Journal of Pharmacognosy and Phytochemistry*, 7(6), 1983-1987.

Sharma, H. K., Sarkar, M., Choudhary, S. B., Kumar, A. A., Maruthi, R. T., Mitra, J., & Karmakar, P. G. (2016). Diversity analysis based on agro-morphological traits and microsatellite-based markers in global germplasm collections of roselle (*Hibiscus sabdariffa* L.). *Industrial Crops and Products*, 89, 303-315.

Shobana, S., Sreerama, Y. N., & Malleshi, N. G. (2009). Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. *Food chemistry*, *115*(4), 1268-1273.

Sneath, P.H. & Sokal, R.R. 1973. Numerical Taxonomy. W.H. Freeman and Company, San Francisco.

Siles, M. M., Russell, W. K., Nelson, L. A., Baltengsperger, D. D., Johnson, B., Van, V. L. D., Jensen S. G. and Hein. G. L. (2004). Heterosis for grain yield and other agronomictraits in foxtail millet. Faculty Papers and Publications in Animal Science. Paper 156.

Sokal, R. R. (1958). A statistical method for evaluating systematic relationships. *Univ. Kansas, Sci. Bull.*, *38*, 1409-1438.

Sonali, S., Shikha, Y., Ramesh, K., Sushma, S., & Neeru, R. (2019). Multi trait analysis reveals substantial diversity in pearl millet [*Pennisetum glaucum* (L.) R. Br.] inbred lines. *Journal of Experimental Biology and Agricultural Sciences*, 7(4), 358-375.

Tomar, M., Bhardwaj, R., Kumar, M., Singh, S.P., Krishnan, V., Kansal, R., Verma, R., Yadav, V.K., Ahlawat, S.P., Rana, J.C., Bollinedi, H. (2021) Nutritional composition patterns and application of multivariate analysis to evaluate indigenous Pearl millet (*Pennisetum glaucum* (L.) R. Br.) germplasm. *Journal of Food Composition and Analysis*, 103: 104086,

566 Upadhyaya, H. D., Reddy, K. N. and Gowda, C. L. L. (2007). Pearl millet germplasm at
567 ICRISAT genebank – status and impact. SAT eJournal, 3:1-5.

568 Velu, G., Rai, K. N., Muralidharan, V., Kulkarni, V. N., Longvah, T., & Raveendran, T. S.
569 (2007). Prospects of breeding biofortified pearl millet with high grain iron and zinc
570 content. *Plant Breeding*, 126(2), 182-185.

571 Vengadessan, V., Rai, K. N., Kannan Bapu, J. R., Hash, C. T., Bhattacharjee, R., Senthilvel, S.,
572 ... & Nepolean, T. (2013). Construction of genetic linkage map and QTL analysis of
573 sink-size traits in pearl millet (*Pennisetum glaucum*). *International Scholarly Research*
574 *Notices*, 2013(1):471632

575 Warrier, S. R., Patel, B. C., Kumar, S., & Sherasiya, S. A. (2020). Combining ability and
576 heterosis for grain minerals, grain weight and yield in pearl millet and SSR markers-
577 based diversity of lines and testers. *Journal of King Saud University-Science*, 32(2),
578 1536-1543.

579 Yadav, O. P., Gupta, S. K., Govindaraj, M., Sharma, R., Varshney, R. K., Srivastava, R. K., &
580 Mahala, R. S. (2021). Genetic gains in pearl millet in India: insights into historic
581 breeding strategies and future perspective. *Frontiers in Plant Science*, 396.

582 Yadav, O. P., Rai, K. N., Khairwal, I. S., Rajpurohit, B. S., & Mahala, R. S. (2011). Breeding
583 pearl millet for arid zone of north-western India: Constraints, opportunities and
584 approaches. *All India coordinated pearl millet improvement project, Jodhpur, India*, 28.

585 Yadav, O. P., Rai, K. N., Yadav, H. P., Rajpurohit, B. S., Gupta, S. K., Rathore, A. and Karjagi,
586 C. G. (2016). Assessment of diversity in commercial hybrids of pearl millet in India.
587 *Indian J. Plant Genet.Resour*., 29(2), 130-136.

588 Zala, H. N., Kulkarni, K. S., Bosamia, T. C., Shukla, Y. M., Kumar, S., Fougat, R. S., & Patel,
589 A. (2017). Development and validation of EST derived SSR markers with relevance to
590 downy mildew (*Sclerospora graminicola* Sacc.) resistance in pearl millet [*Pennisetum*
591 *glaucum* (L.) R. Br.]. *Journal of Plant Biochemistry and Biotechnology*, *26*(3), 356-
592 365.