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ORIGINAL ARTICLE

Genetic dissection of minerals and phytate content in pearl millet germplasm panel using genome-wide association study

Satbeer Singh[1,2] | **Hanna Rose Manwaring**[1,3] | **Aavula Naveen**[1,4] | **Matthew Hegarty**[5] | **Rattan S. Yadav**[1]

[1]Institute of Biological Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth, UK

[2]Division of Agrotechnology, Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research (CSIR), Palampur, Himachal Pradesh, India

[3]School of Water, Energy and Environment, Cranfield Forensic Institute, Cranfield University, Cranfield, UK

[4]Indian Agricultural Research Institute (IARI), Pusa, New Delhi, India

[5]Department of Life Sciences, Aberystwyth University, Aberystwyth, UK

Correspondence
Rattan S. Yadav, Institute of Biological Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth SY23 3EE, UK.
Email: rsy@aber.ac.uk

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Abstract

Billions of people around the world suffer from malnutrition, leading to severe adverse health effects. Pearl millet (*Pennisetum glaucum*) is a multifaceted versatile crop with excellent nutritional profile which can help to combat nutritional disorders and climate change. In this study, we evaluated the global pearl millet germplasm panel known as PMiGAP for natural variation and genetic maker trait associations for important minerals, that is, iron, zinc, calcium, magnesium, potassium and sodium along with phytate. The genotypes IP-15947, IP-5121, IP-4020, IP-12768, IP-5695, IP-8786 and IP-11310 were found to be superior for majority of minerals examined but had lower phytate-to-zinc ratio. Phytate/mineral molar ratios are typically used to predict the bioavailability of iron and calcium contents in grains, and surprisingly none of the PMiGAP genotypes showed such ratios below threshold indicating PMiGAP entries studied in this study seriously suffer from bioavailability issues of these minerals. On the other hand, 73 genotypes had lower zinc/phytate ratio than the threshold in the germplasm panel. Iron and zinc content had significant positive association among them but phytate content in general was not significantly correlated with minerals except for magnesium and potassium. A genome-wide association study using 456 K SNPs identified 74 significant marker–trait associations and 59 candidate genes around 50 Kb distance near the significant SNPs. Ten significant SNPs were found within the candidate genes. The associated markers and the candidate genes provide new insights into the genetic architecture of the mineral traits studied and will facilitate marker-assisted selection to accelerate breeding of such minerals in future varieties to combat rising malnutrition problem via diet.

KEYWORDS

bioavailability, genetic diversity, GWAS, hidden hunger, mineral and phytate contents, PMiGAP

Satbeer Singh and Hanna Rose Manwaring shared first authorship.

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1 | INTRODUCTION

The world today is facing a global nutrition crisis. According to the Global Report on Food Crises (2022), the number of people facing acute hunger reached nearly 193 million in 2021 (https://data.unicef.org/resources/sofi-2021/). Furthermore, according to an estimate by the WHO, around 2.5 billion people globally suffer from nutritional deficiencies and show symptoms of malnutrition (https://www.who.int/news-room/fact-sheets/detail/malnutrition). It is estimated that hidden hunger affects over 2 billion people worldwide, particularly in low- and middle-income countries (Lowe, 2021).

Cereal grains are a rich source of proteins, carbohydrates and nutrients in human nutrition. As good sources of carbohydrates, protein, dietary fibre, minerals, antioxidants and vitamins (Gangashetty et al., 2023; Saleh et al., 2013; Sehgal et al., 2015), millets can contribute to healthy diets in terms of minerals too. Pearl millet grain provides an impressive spectrum of calcium 23–32 mg, copper 0.45–0.54 mg, iron 6.3–15.2 mg, magnesium 84–124 mg, phosphorus 289–427 mg, potassium 291–365 mg, sodium 4–12 mg and zinc 2.6–4.1 mg per 100 g (FAO, 2023). Additionally, consumption of pearl millet also helps in minimizing cancer, type 2 diabetes and celiac disease mainly due to its slow digestive carbohydrate, higher minerals and low gluten properties. These nutritional qualities have promoted use of pearl millet flour in fortification of food to improve nutritional quality of food products.

Pearl millet (*Pennisetum glaucum* (L.) R. Br. and syn. *Cenchrus americanus* (L.) Morrone) is the fifth most important cereal crop cultivated across the world next to rice, wheat, maize and sorghum (Satyavathi et al., 2021). It is a cross-pollinating species with a protogynous blooming pattern and exhibits a C4 photosynthetic pathway. It can be cultivated in the most unfavourable agroecologies where other cereals, such as rice or wheat, do not thrive and provide an opportunity for diversified cropping systems. Because of short developmental stages, fast growth rate and high photosynthetic efficiency, it is an ideal crop for shorter growing seasons and has built-in adaptation to low-fertility soils (Serba et al., 2020).

The human body needs about 20 essential elements to function properly and among them, sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) are the most important ones. Calcium forms the basic structural framework of teeth and bones. Magnesium functions as a cofactor for over 300 enzymes, it plays a pivotal role in regulating fundamental physiological processes, including muscle contraction, neuromuscular transmission, glycaemic control, myocardial contraction and blood pressure (Gröber et al., 2015). Together with potassium, the active ionized form of sodium performs a number of vital functions that ensure the homeostatic state of the body (Kodintsev et al., 2022). Iron and zinc are the most important trace elements with specific biochemical functions in human body (Godswill et al., 2020). Thus, micronutrient deficiencies termed hidden hunger can lead to increased morbidity and mortality in the elderly, low birth weight and developmental issues in babies and stunting and cognitive impairments in children. Biofortification of staple crops stands out as the most cost-effective strategy to address this issue effectively (Singh et al., 2021). However, biofortification outcomes in pearl millet are also limited by the presence of antinutrients like phytate. Phytate is the salt of phytic acid, *myo*-inositol-1,2,3,4,5,6 hexa*kis*-phosphate; it is widely distributed in the plant kingdom and serves as the major form of stored phosphorus and minerals containing up to 75% of total phosphorus in the kernel (Boncompagni et al., 2018).

Although there is significant potential, a comprehensive study has not yet been undertaken to explore the range of genetic variations in global pearl millet germplasm, particularly for macro- and micro-nutrient concentrations along with key antinutrient phytate. Understanding such variations and genetic diversity is crucial for their effective incorporation in pearl millet breeding programmes. GWAS mapping has proven to be a powerful tool for unravelling the genetic basis of quantitative traits in plants compared to conventional QTL mapping (Sehgal et al., 2015). Availability of genetic and genomic resources such as pan-genome sequence (Ramu et al., 2023; Varshney et al., 2017) and creation of Pearl Millet Inbred Germplasm Association Panel (PMiGAP) (Sehgal et al., 2015; Yadav et al., 2022) are now making it possible to identify candidate genes for global traits across the genome. However, until now, not many traits have been genetically dissected in pearl millet at the germplasm level. Considering the above research gap, the present study was planned to identify marker–trait associations and candidate genes underlying the natural variation for important minerals using a subset of 229 pearl millet accessions randomly selected from a world collection of 345 accessions known as PMiGAP (Sehgal et al., 2015).

2 | MATERIALS AND METHODS

2.1 | Plant material

The set of pearl millet accessions used in this study comprised 229 accessions randomly drawn from within the Pearl Millet Inbred Germplasm Association Panel (PMiGAP), which represents pearl millet genetic diversity from across the world (Sehgal et al., 2015). The 229 genotypes included in this study belonged to 22 different

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countries including 55 accessions from India, 22 from Niger, 10 from Nigeria, 9 each from Namibia, Zimbabwe and Togo and the remaining 52 from other different locations (Table S1). Of 55 accessions from India, proportionally the largest number of genotypes (40 accessions) were from ICRISAT, a global centre of excellence in pearl millet breeding research. Other genotypes included were from African countries commonly used as parents in pearl millet breeding across the globe. Seeds of each of the 229 accessions were multiplied at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru (India), under uniform field conditions as described in earlier reports (Ramya et al., 2018; Upadhyaya et al., 2008). For multiplication, each genotype was grown in a plot of three rows at 75 cm spacing between rows and 15 cm between the plants in a row. Thinning was done to maintain the single plant per drill. A basal dose of 100 kg/ha of di-ammonium phosphate was applied. Two manual weeding were done to keep the plot weed free. Cluster bagging, sibbing and selfing techniques were followed to maintain the genetic integrity of the genotypes (Upadhyaya et al., 2008).

2.2 | Estimation of various minerals and phytate components

One gram of whole pearl millet seeds from each genotype was ground to a fine powder using an electric-powered grinding mill, which was able to pass through a 1 mm sieve. Then, each 1 g of sample was digested into a 100 mL Kjeldahl flask with 15 mL digestion acid (Aqua regia, 780 mL of concentrated hydrochloric acid + 500 mL nitric acid + 7.20 mL deionized water) overnight followed by heating at 120°C for 3 h and cooled for a further 1 h. The solution was washed into a 50 mL volumetric flask using diluent acid (250 mL nitric acid + 2 L deionized water) and shaken. The contents of each volumetric flask were filtered through Whatman No. 1 filter paper (25 mm). Then, the multielement analysis for minerals including iron (Fe), zinc (Zn), calcium (Cu), magnesium (Mg), potassium (K) and sodium (Na) was carried out using inductively coupled plasma optical emission spectroscopy (ICPOES, Optima 8000DV, PerkinElmer, USA).

Phytate content was determined as total phosphorus using the phytic acid assay kit (K-PHYT, Megazyme International Ireland Limited, Bray, Ireland) (Manwaring, 2018). In brief, separately, 0.5 g of whole pearl millet seed from each genotype was ground and passed through a 1 mm sieve for phytate estimation. This was transferred into a 75 mL glass beaker and 20 mL 0.66 M hydrochloric acid was added. The sample was left to stir overnight. One microlitre of the extract was centrifuged at 15210 *g* for 10 min and 0.5 mL of the supernatant was transferred to a fresh 1.5 mL microfuge tube. The sample was then neutralized by the addition of 0.5 mL sodium hydroxide solution (0.75 M). Then, the enzymatic dephosphorylation was carried out in 1.5 mL centrifuge tubes with phytase (buffer + sodium azide + phytase suspension, pH 5.5, in a water bath set to 40°C for 10 min) followed by alkaline phosphatase (buffer + MgCl2 + ZnSo4 + sodium azide + alkaline phosphatase suspension, in a water bath set at 40°C for 15 min). After 15 min, the reaction was stopped by addition of trichloroacetic acid (50% w/v) and centrifuged at 13,000 rpm for 10 min, the supernatant was extracted and used for the colorimetric determination of phosphorus. A phosphorus calibration curve was performed concurrently using the same batch of colour reagent (ammonium molybdate, 5% w/v added to ascorbic acid and 10% w/v/1 M sulphuric acid).

2.3 | Statistical analyses

A descriptive statistical analysis was conducted to summarize the frequency distribution of variables among the pearl millet accessions. Correlation studies, Pearson's correlation coefficients and principal component analysis (PCA) were executed using R Studio (version 4.3.2) (Posit Team, 2023). The grouping pattern was obtained with K-means clustering algorithm using 'kmeans' function under package 'factoextra' in R Studio which partitioned all the observations into k clusters with the nearest mean (Ikotun et al., 2023). To determine the optimal number of clusters for K-means clustering, we compared plots generated by the Elbow method using function 'wss' and average Silhouette method using function 'silhouette' under the package 'factoextra' in R Studio.

2.4 | GWAS analysis

The re-sequencing data of all the genotypes were derived from the publicly available dataset (Ramu et al., 2023; Varshney et al., 2017). A final set of 456 K SNPs were filtered with minor allele frequency of more than 0.05, and missing rate of less than 10% in PLINK (Purcell et al., 2007). The genome-wide association analysis was carried out using multilocus fixed and random model circulating probability unification (FarmCPU) model in Genome Association and Prediction Integrated Tool (GAPIT) function on R Studio (Wang & Zhang, 2021). A threshold for the significance of SNP markers was set at p-value < 0.00001 ($-\log10p \geq 5.0$). However, to reduce the false positive at maximum capacity for the traits where a large number of positives were observed even at

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$-\log10p > 4.0$, the cutoff level for significant markers was established using a modified multiple-test Bonferroni's correction (Kimani et al., 2020). The SnpEff eff (Version 4.3.1 t) package (Cingolani et al., 2012) was used to detect candidate genes on the *Pennisetum glaucum* reference genome (Tift23D2B1-P1-P5) within regions around 50 kb around significant SNPs to avoid the expected linkage disequilibrium (LD) decay in this highly cross-pollinated crop. Using the SnpEff eff package, a custom database was constructed using the GFF (general feature format) file of the 'Tift' reference genome (Tift23D2B1-P1-P5). The upstream and downstream regions surrounding each strongly linked SNP were investigated in order to identify and propose potential candidate genes. In addition, each region of interest with potential candidate genes was subjected to a Basic Local Alignment Search Tool (BLAST) search of the NCBI database against the National Center for Biotechnology Information (NCBI) nr database.

3 | RESULTS

3.1 | Extent of mineral diversity in PMiGAP

The comprehensive analysis of variance (Table S2) and descriptive statistics, including mean, range and coefficients of variation, demonstrated a wide spectrum of variation among the PMiGAP genotypes for all examined traits, as detailed in Table 1. The frequency distribution curves for these genotypes predominantly exhibited normal to near-normal patterns for most traits, with the notable exception of sodium (Na) (Figure 1). In terms of mineral content within this germplasm, the observed hierarchy from highest to lowest was potassium (253.90 to 534.60 mg/100 g) > magnesium (84.50 and 179.60 mg/100 g) > iron (21.90 and 112.80 mg/kg) > zinc (20.72 to 71.11 mg/kg) > calcium (12.88 to 46.36 mg/100 g) > sodium (1.50 to 15.51 mg/100 g). The antinutrient phytate levels varied from 0.25 to 1.30 g/100 g, with an average value of 0.75 g/100 g. All genotypes were compared with population mean of each trait and a list of the superior genotypes is given in Table 2, which were simultaneously higher for multiple minerals and lower phytate as compared to the overall population mean.

Iron (Fe) and zinc (Zinc) showed highest positive correlation ($r = 0.74$, $p < 0.001$) among the studied minerals. Zinc also correlated positively with magnesium (Mg) ($r = 0.24$, $p < 0.001$) but negatively with potassium (K) ($r = -0.18$, $p < 0.01$), similar to Fe. Numerous other significant correlations existed but were comparatively weaker, including positive Ca-K, Ca-Mg, Ca-Na, Ca-Fe, Mg-Ca, Mg-K, Mg-PA, Mg-Zn and K-Na associations along with negative K-Zn and K-Fe associations. Essentially, iron and zinc levels were strongly related, while calcium, magnesium and sodium showed multiple moderate correlations across traits. Meanwhile, potassium was negatively correlated with iron and zinc while positive with calcium, magnesium and sodium content. While further investigation into the intricate interplay among these grain minerals is justified, the data clearly shows that specific mineral concentrations exhibit tight inherent correlations in pearl millet.

Phytate acts as major antinutritional factor in pearl millet because it forms complexes with minerals, thereby reducing their bioavailability. Phytate exhibited a non-significant correlation with Fe, Zn, Na and Ca, indicating the potential for independent improvement of these traits without mutual interference. Phytate showed significant positive correlations with K ($r = 0.15$, $p < 0.001$) and Mg ($r = 0.31$, $p < 0.001$), which is not desirable during improvement of bioavailability of these minerals.

3.2 | Patterns of mineral diversity in PMiGAP

To gain a deeper insight into the data structure, dimensionality, distribution and relationships of traits across the PMiGAP germplasm, principal component analysis and K-mean clustering were carried out (Figure 2). The major contributors to variation observed in first principal

TABLE 1 Mean, range and phenotypic variation for various minerals and phytate content in PMiGAP.

Characters	Mean	Min	Max	SD	CV (%)	Standard error
Ca (mg/100 g)	22.40	12.88	46.36	5.76	25.73	0.38
K (mg/100 g)	349.49	253.90	534.60	46.19	13.22	3.01
Mg (mg/100 g)	123.31	84.50	179.60	12.66	10.27	0.83
Na (mg/100 g)	3.86	1.50	15.51	1.90	49.35	0.12
Fe (mg/kg)	46.61	21.90	112.80	13.50	28.96	0.88
Zn (mg/kg)	42.62	20.72	71.11	9.36	21.96	0.61
Phytate (g/100 g)	0.75	0.25	1.30	0.18	24.43	0.01

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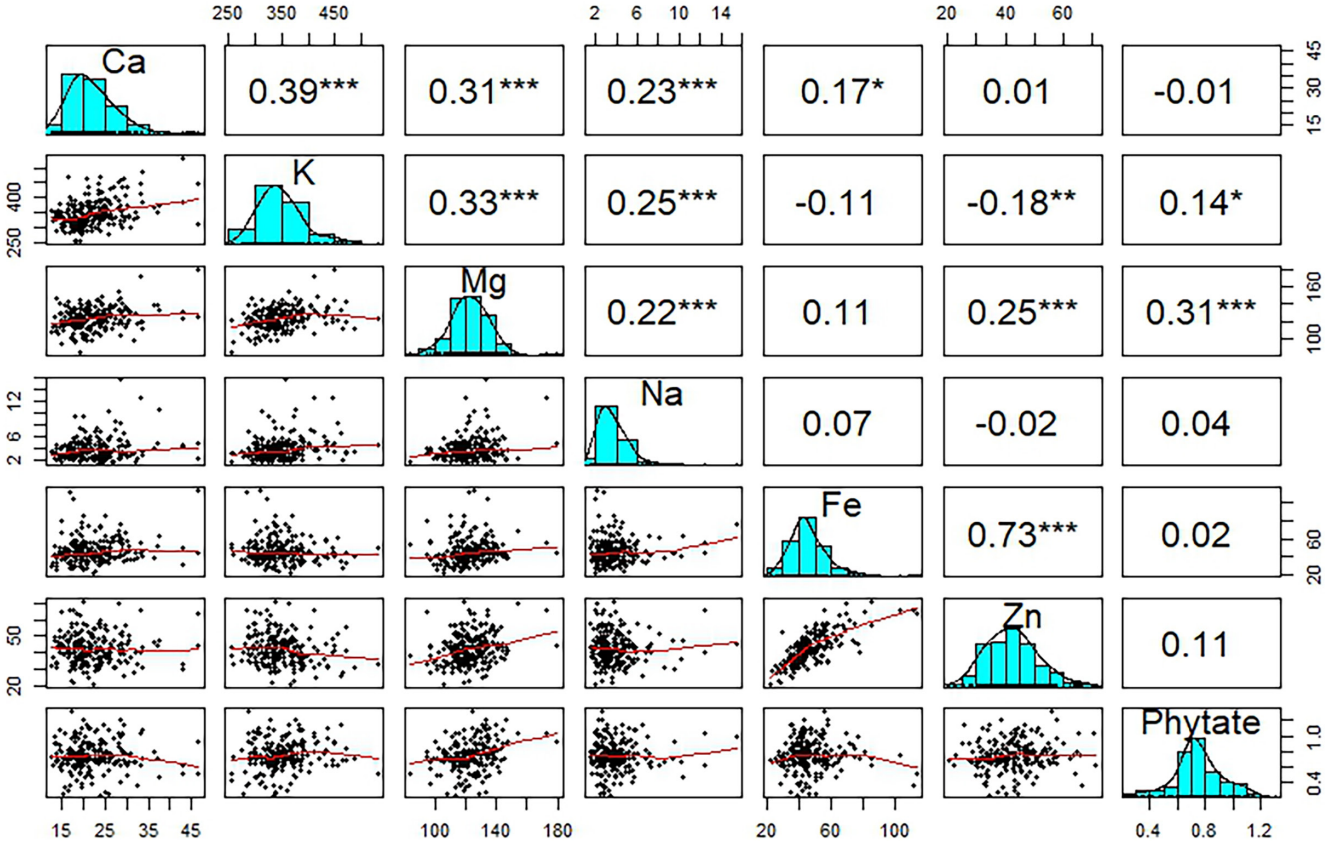

FIGURE 1 Distribution (below diagonal) and density curve-enhanced histograms (diagonal) illustrating patterns of grain minerals and phytate components across 229 genotypes. Above diagonal values depict Pearson's correlation coefficients (*r*) among studied traits. Asterisks (*, **, ***) indicate the significance levels of the correlation coefficients at different alpha values (0.05, 0.01 and 0.001 respectively).

TABLE 2 List of genotypes showing higher values for majority of minerals and lower values for phytate as compared to the overall population mean.

Genotype	Ca (mg/100 g)	K (mg/100 g)	Mg (mg/100 g)	Na (mg/100 g)	Fe (mg/kg)	Zn (mg/kg)	Phytate (g/100 g)
IP-15947	37.39	375.6	132.8	10.41	62.9	47.07	0.4722
IP-5121	37.15	351.7	139.4	4.81	52.45	44.25	0.7055
IP-4020	27.38	403.1	138	7.13	62.33	55.52	0.6478
IP-12768	30.33	434.5	118.1	5.13	57.89	45.11	0.629
IP-5695	28.68	456.6	121.3	8.12	60.48	51.65	0.6161
IP-8786	24.94	357.2	123.9	2.48	46.62	47.25	0.7318
IP-11310	23.83	445.6	122.2	4.64	49.63	47.93	0.6874

component were Mg, Ca, Fe and Zn. The major contributors to variation observed in second principal component were Zn, Fe and K. The results showed that certain minerals were particularly important in differentiating among studied genotypes. The observed significant positive association among various minerals provides valuable insights into the potential for concurrent enhancement of these mineral levels in pearl millet, suggesting promising avenues for nutritional improvement in this crop.

The genotypes within the Pearl Millet Inbred Germplasm Association Panel (PMiGAP) were categorized into three distinct ellipses, namely breeding lines, improved cultivars and land races that were almost overlapped (Figure 2a). This indicated existence of consistent and similar patterns of variability for mineral contents from landraces to improved cultivars. These cultivars can be explored further for including in pre-breeding and modern breeding programmes for biofortification. There is no strict relationship observed between clustering pattern of genotypes and their eco-geographical distribution. The K-means clustering categorized the germplasm into three clusters (Figure 2b). The inter-cluster distance values

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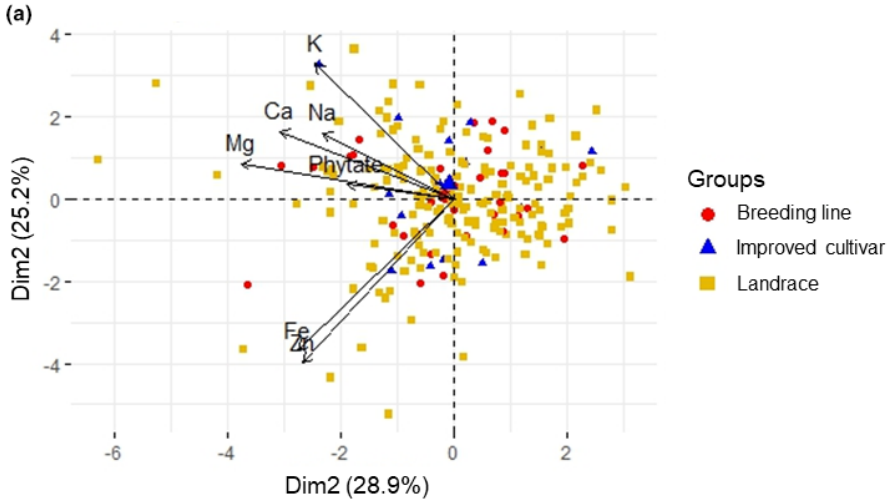

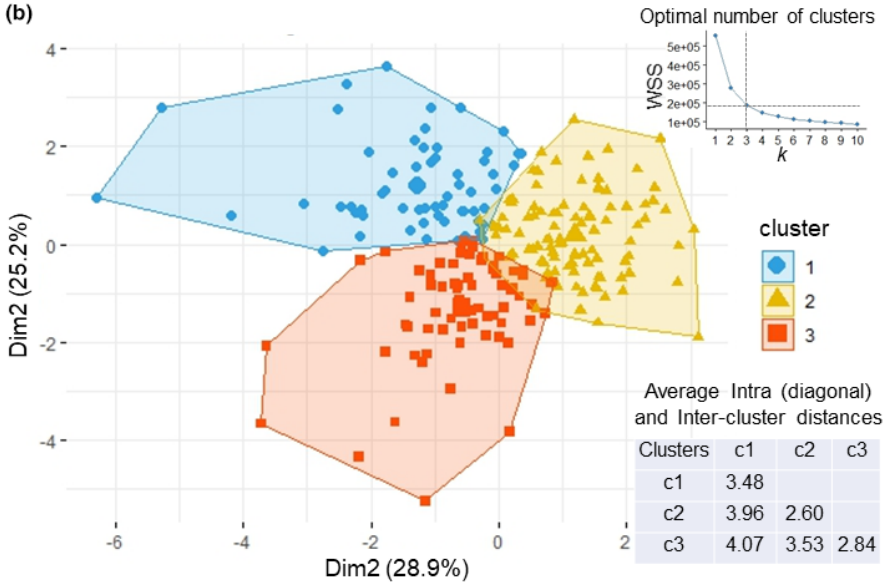

FIGURE 2 PCA biplot (a) displaying the distribution of Pearl Millet Inbred Germplasm Association Panel (PMiGAP) for studies traits based on type of genotype (breeding line, improved cultivar and landrace). The clustering biplot (b) depicts k-mean clustering of all the genotypes.

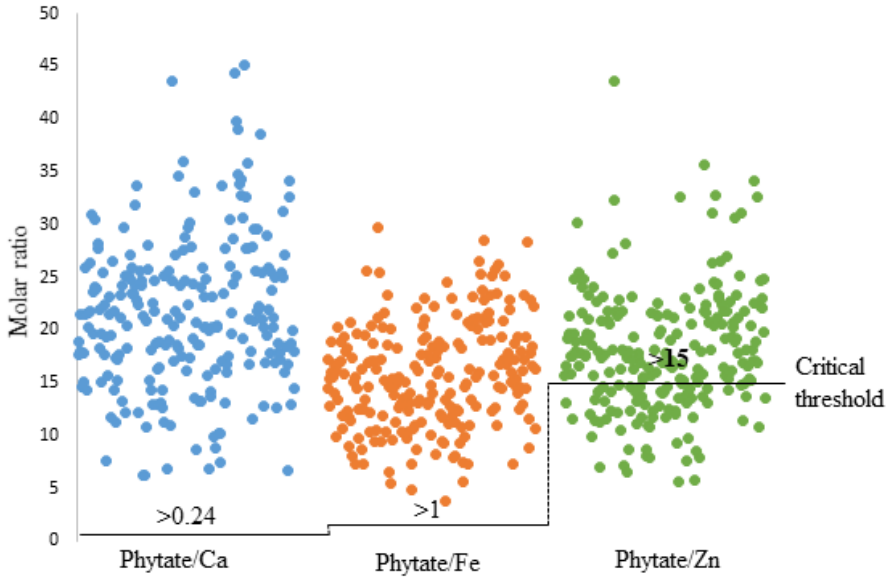

FIGURE 3 Phytate/Ca, phytate/Fe and phytate/Zn molar ratios to predict bioavailability of minerals in PMiGAP.

ranged from 3.53 to 4.07. Cluster I versus III had the largest inter-cluster distance (4.07). The genotypes found in these clusters had a wide range of genetic diversity and might be employed in a peal millet hybridization programme to recover better transgressive segregates, aiding the production of high-yielding varieties. The smallest inter-cluster

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distances were found between clusters II versus III (3.53), demonstrating a close association between these clusters. Therefore, for any hybridization programmes in pearl millet, the choice of suitable diverse parents based on genetic divergence analysis would be more rewarding than the choice based on geographical distances.

3.3 | Bioavailability of minerals in PMiGAP

The bioavailability of minerals is strongly affected by the levels of phytate content and could be predicted on the basis of phytate/mineral molar ratios. The molar ratios exceeding the critical values indicate the proportion of the population that is likely to have reduced mineral bioavailability. In the present investigation, phytate/Ca, phytate/Fe and phytate/Zn molar ratios range from 6 to 45, 3 to 28 and 6 to 44 respectively. Astonishingly, all the PMiGAP entries had higher phytate/Fe and phytate/Ca molar ratios than their critical value of 1.0 and 0.24, respectively, which indicated that this panel is likely to have reduced Fe and Ca bioavailability (Figure 3). However, there were 77 genotypes that fall below the critical value for Zn (<15), which indicated that the Zn bioavailability is higher in these genotypes. Interestingly, 70% of these genotypes are of African origin, which may suggest that lines from this region may naturally have a lower phytate/Zn molar ratio, as compared to Indian lines.

3.4 | Marker–trait associations

A total of 456,156 SNPs spanning 1783 Mb were used for GWAS analysis, representing approximately 99.6% of the 1.79 Gb pearl millet genome. Among all seven chromosomes, chromosome 2 had the maximum number of SNPs (84,616) while chromosome 7 had the lowest (50,683), resulting in an average SNP density of 256 SNPs per Mb (1 SNP per 3.9 Kb). Details on SNP distributions across each chromosome are provided in Table S3. The LD decay in the studied population remained at 50% at around 107Kb, which is quite higher than the previously reported studies in pearl millet with 994 accessions (Varshney et al., 2017), comparable to another study that included 398 accessions (Serba et al., 2019), but lower than reported in sorghum (Kimani et al., 2020), wheat and rice (Wang et al., 2023). The number of SNPs we used in the current study was much higher than suggested by the LD decay. Manhattan plots illustrate the markers significantly associated with the studied traits across all chromosomes (Figure 4a). Marker–trait associations were identified using the FarmCPU model for all studied traits across chromosomes. The Q-Q plots depict observed marker–trait associations compared to expected associations after accounting for population structure (Figure 4b).

Genome-wide association analysis uncovered a total of 74 highly significant marker–trait associations (MTAs) associated with grain mineral concentrations at a significance threshold of $p < 0.00001$ ($-\log_{10}p \geq 5$). Our analysis detected MTAs distributed across the majority of chromosomes for minerals as follows: 17 MTAs for potassium (K) on chromosomes 1, 2, 4, 5, 6 and 7; 16 MTAs for sodium (Na) on chromosomes 1–6; 15 MTAs for calcium (Ca) on chromosomes 1–6; 15 MTAs for iron (Fe) on all 7 chromosomes; 10 MTAs for zinc (Zn) on chromosomes 4 and 5; and 1 MTA for magnesium (Mg) on chromosome 5. More details on these specific MTAs can be found in Table S4 and Figure S1. Using a more stringent Bonferroni correction ($-\log_{10}p \geq 6$), 34 robust MTAs were found: 1 for calcium (Ca); 12 for potassium (K); 10 for sodium (Na); 10 for iron (Fe); and 1 for zinc (Zn). We also identified one pleiotropic SNP on chromosome 1 (Chr01-179327483) exhibiting shared associations with both potassium (K) and calcium (Ca), indicating genetic effects across multiple traits.

3.5 | Candidate genes

In this study, we identified a total of 59 candidate genes around 50 kb distance from a significant SNP (Table S5), including 10 candidate genes that have significant marker SNPs within them (Table 3). These genes are involved in the accumulation of various minerals in pearl millet grains, including iron (Fe), potassium (K), sodium (Na), calcium (Ca) and zinc (Zn). Specifically, three candidate genes were found for Fe, two for K, three for Na, one for Ca and one for Zn. These genes have diverse biological and molecular functions related to mineral uptake, transport and metabolism in pearl millet.

The SNP associated with Ca accumulation was linked to the gene dpca4g283950.840, which encodes a protein containing Brix domains. For Fe, linked genes were as follows: dpca2g097300.840 encoding a protein with EMP24_GP25L domains; dpca5g349180.840 encoding a serrate RNA effector molecule-like protein containing various domains including AAA_16 and Rx_N; and dpca5g365440.840 encoding a protein with DNA_pol_B_N and SAP domains. The K-linked genes were as follows: dpca1g045590.840 encoding an uncharacterized glycohydrolase protein with Glyco_hydro_1 domains and dpca5g368200.840 encoding a predicted transmembrane protein 184C isoform X1 with Solute_trans_a domains. The Na-associated gene was dpca2g089970.840, encoding a C2H2-type zinc finger protein with multiple zinc finger domains. Finally, the Zn SNP was linked to dpca5g337680.840, encoding a hypothetical protein containing DUF1475 and DUF2644 domains.

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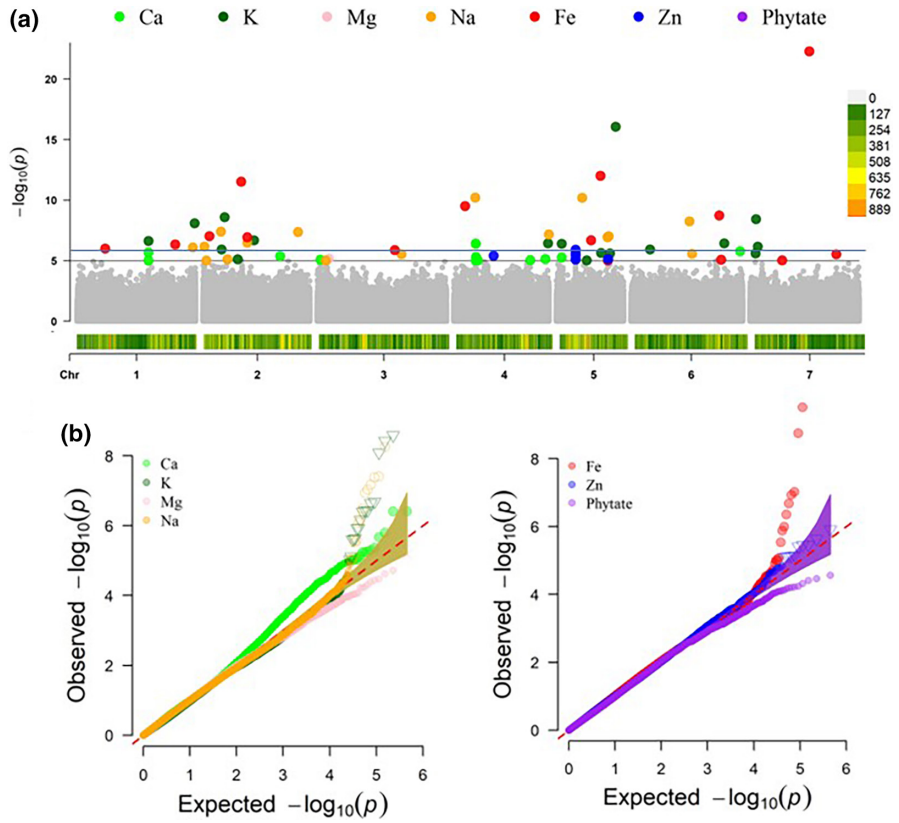

FIGURE 4 Manhattan plot (a) and Q-Q plots (b) show marker–trait associations for all the seven traits studied using 456 K SNPs. The bottom of Manhattan plot showed distribution of SNPs/Mb as per the legend scale presented on right side. The upper level of threshold was set using Bonferroni correction for strong associations.

4 | DISCUSSION

Human body requires at least 49 different nutrients to fulfil metabolic requirements. Among those, insufficient intake of key minerals, including magnesium (Mg) for cardiovascular health, calcium (Ca) for osteoporosis prevention and potassium (K) for avoiding hypokalaemia, can lead to various health issues (Govindaraj, Yadav, et al., 2020). Also, iron and zinc deficiencies are a serious public health problem in developing countries (Satyavathi et al., 2022). Superior genotypes from PMiGAP for multiple minerals are identified in our study. Genotypes IP-15947IP-5121, IP-4020, IP-12768, IP-5695, IP-8786 and IP-11310 were found superior (above overall mean) for all traits studied. The significant and positive correlations between Fe and Zn concentrations indicate the effectiveness of concurrent selection for Fe and Zn in pearl millet (Govindaraj, Rai, et al., 2020; Govindaraj, Yadav, et al., 2020). Moreover, improvement for high Fe and Zn is not likely to reduce the level of other mineral elements in pearl millet. Non-significant correlation of phytate with Fe, Zn, Na and Ca indicated that reducing phytate will not affect these minerals.

As indicated by the Harvest plus programme (https://www.harvestplus.org), the global baseline for iron content in non-biofortified varieties is set to 47 mg/kg, while in iron-biofortified varieties, it is 77 mg/kg. Interestingly, in our study, seven genotypes IP-9446, IP-9301, IP-8955, IP-9406, IP-16638, IP-9282 and IP-7536 were identified that surpassed the target concentration of iron in iron-biofortified varieties. These seven genotypes provide an opportunity to be used as donors for iron enrichment in improved pearl millet varieties. Eighty-eight genotypes surpassed the baseline iron concentration in non-biofortified varieties which is 47 mg/kg. These results indicate the potential of this panel for use in mineral enrichment through pre-breeding and biofortification programmes. In India, since 2018, benchmark levels adopted for Fe and Zn in pearl millet cultivar release policy were 42 and 32 ppm respectively. Notably, from this PMiGAP, 143 genotypes are surpassing the limits for iron. Similarly, 207 genotypes surpassed the limits for zinc showcasing the richness of minerals in this panel. Selection in this germplasm may be more effective for Mg, Ca, Fe and Zn as they are the major contributors to variation observed in first principal component. These selected accessions can be directly utilized, especially if they exhibit superior agronomic traits. Alternatively, they can serve as valuable donors in the creation of elite pearl millet varieties that combine high yield and elevated mineral concentrations. This can be achieved through both conventional breeding methods and advanced genomic-assisted approaches. The diversity patterns over different types of accessions (breeding lines, cultivars and landraces) were fully overlapped. This observation aligns with findings by Sehgal et al. (Sehgal et al., 2015), who identified six

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TABLE 3 Candidate genes presented around associated marker SNPs.

Traits	SNPs	Chr	Positions	*p*-value	Gene	Function
Ca	Chr01-179327483	4	60591660	9.3E-06	dpca4g283950.840	Protein containing Brix domains
Fe	Chr02-87179735	2	15828724	9.5E-08	dpca2g097300.840	Protein containing EMP24_GP25L domains
Fe	Chr02-239038728	5	87109850	2.1E-07	dpca5g349180.840	Predicted: serrate RNA effector molecule-like (gfam002777) | Protein containing AAA_16, AAA_22, ARS2, DUF3546, LRR_4, LRR_8, NB-ARC and Rx_N domains
Fe	Chr03-7440421	5	129777323	9.9E-06	dpca5g365440.840	Protein containing DNA_pol_B_N, SAP domains
K	Chr04-28192878	1	179327483	2.3E-07	dpca1g045590.840	Uncharacterized protein (gfam000115) | Protein containing Glyco_hydro_1 domains
K	Chr04-55468669	5	135179689	2.4E-06	dpca5g368200.840	Predicted: transmembrane protein 184C isoform X1 (gfam000522) | Protein containing Solute_trans_a domains
Na	Chr05-48622528	2	2851059	6.8E-07	dpca2g089970.840	C2H2-type zinc finger protein ZFP36 (gfam000388) | Protein containing zf-C2H2, zf-C2H2_4, zf-C2H2_6 and zf-C2H2_jaz domains
Na	Chr05-48626366	2	7956767	9.6E-06	dpca2g093240.840	Predicted: ubiquitin-conjugating enzyme E2 N (gfam000034) | Protein containing UQ_con domains
Na	Chr05-64826513	2	111176959	3.2E-07	dpca2g134450.840	Predicted: type I inositol 1,4,5-trisphosphate 5-phosphatase 2 isoform X1 (gfam000300) | Protein containing Exo_endo_phos domains
Zn	Chr06-47909910	5	48303792	3.5E-06	dpca5g337680.840	Hypothetical protein (gfam059769) | Protein containing DUF1475 and DUF2644 domains

subpopulations within the PMiGAP comprising 345 entries. Furthermore, there was no observed association between the clustering pattern and the eco-geographical distribution of the genotypes.

The absorption of Zn and Fe from a meal corresponds directly to its phytate levels (Al Hasan et al., 2016). Phytate/mineral molar ratios are typically used to predict the inhibitory effect of phytate on the bioavailability of minerals based on a 'critical value'. For example, if the phytate/Fe molar ratio is >1, this is indicative of poor Fe bioavailability (Hallberg et al., 1989), as well as if phytate/Zn molar ratio is >15 showed poor availability of Zn (Turnlund et al., 1984) and phytate/Ca ratio > 0.24 is for poor calcium availability (Ma et al., 2007). All the PMiGAP entries had reduced Fe and Ca bioavailability. However, 73 lines had higher bioavailability of Zn, which could be used in crossing programmes for the creation of elite lines with low phytate/Zn molar ratio. Studies in wheat and rice showed the phytate/Zn ratio was almost equivalent and more than 15 (Li et al., 2015). Soaked grains of sorghum and maize showed reduced phytate/Zn molar ratios (Kruger et al., 2014).

Furthermore, biofortification efforts can be accelerated by identifying and incorporating QTLs/genes linked to grain mineral content through marker-assisted breeding. The identification of candidate genes associated with mineral traits in pearl millet provides valuable insights into the genetic basis of mineral accumulation in this important crop. While some studies have reported genes related to iron and zinc content in grains (Anuradha et al., 2017; Goud et al., 2022; Kumar et al., 2018; Pujar et al., 2020; Satyavathi et al., 2022), other essential minerals like calcium, magnesium, potassium and sodium as well as phytate content have often been overlooked. The candidate genes identified around the significant SNPs in this study are involved in a variety of biological and molecular functions, indicating the complexity of mineral accumulation in pearl millet (Hepler, 2005). The protein containing Brix domain near Ca-related SNPs may be involved in

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Ca uptake, transport and metabolism through ribosome biogenesis and RNA processing in pearl millet grains (Prigge & Ry Wagner, 2001). The EMP24_GP25L domains aid protein trafficking and secretion in cells (Lopez et al., 2020). Also, EMP24 is involved in the splicing of mitochondrial introns, complex I assembly and seed development in maize indicating its role in mitochondrial RNA processing (Xiu et al., 2020). Hence, the pearl millet protein with these domains may likely contribute to Fe transport or storage in grains. The predicted serrate RNA effector molecule-like protein containing AAA_16, AAA_22, ARS2, DUF3546, LRR_4, LRR_8, NB-ARC and Rx_N domains suggests that this protein may be involved in RNA processing or regulation, which could indirectly affect iron metabolism in the plant. This protein is expressed in shoot meristems and emerging organ primordia throughout development. Its mutant displays defects in shoot and leaf development or death during embryogenesis in Arabidopsis (Prigge & Ry Wagner, 2001). While the exact function of the protein containing DNA_pol_B_N and SAP domains in relation to iron accumulation is not clear, the presence of these domains suggests that it may be involved in the synthesis of iron-binding proteins (Fatima et al., 2021). For potassium (K), the presence of Glyco_hydro_1 domains in the protein suggests that this gene may be involved in the breakdown of complex carbohydrates in the cell wall, releasing potassium ions for uptake by the plant. Proteins containing Glyco_hydro_1 domains play significant roles in plants, particularly in the breakdown, biosynthesis or modification of glycosidic bonds present in carbohydrates and glycoconjugates (Bradley et al., 2022). This process is important for maintaining potassium homeostasis and ensuring proper plant growth and development. Similarly, the presence of Solute_trans_a domains in dpca5g368200.840 suggests that this gene may be involved in the transport of potassium ions across membranes within the plant (Yao et al., 2020).

A protein named C2H2-type zinc finger protein ZFP36, which contains zf-C2H2, zf-C2H2_4, zf-C2H2_6 and zf-C2H2_jaz domains was found associated with sodium (Na). It may be possible that ZFP36 regulates the expression of genes involved in sodium uptake, transport or storage in pearl millet (Li et al., 2022). However, in the context of the gene dpca5g337680.840, which encodes a protein with DUF1475 and DUF2644 domains, the exact function of these domains remains unclear. They may be involved in protein–protein interactions or other cellular processes related to zinc metabolism. Further functional validation studies are needed to elucidate the exact roles of these genes in mineral accumulation in pearl millet.

5 | CONCLUSION

In this paper, we report for the first-time diversity patterns in global pearl millet germplasm collection for minerals and phytate contacts, and also of their genetic dissection using GWAS. The study identified seven superior genotypes for most mineral traits from within the PMiGAP which also surpasses the minimum iron levels set by the Harvest plus towards biofortification. These identified accessions therefore can be directly used as donors in pearl millet breeding programme in developing mineral-dense and low antinutrient varieties. Moreover, markers and candidate genes associated with increased minerals have been identified to use in transferring these traits into popular cultivars via marker-assisted breeding. The 74 significant marker–trait associations and 59 candidate genes are reported in this study which would facilitate marker-assisted selection approaches to develop biofortified crop varieties with speed and precision. Although these findings are of significant importance, our study was conducted on seeds harvested from a single environment and Genotype x Environment interaction on the SNPs and the candidate genes reported need further investigations.

AUTHOR CONTRIBUTIONS

HRM, project implementation, data generation and biochemical analysis; SS, data analysis and original draft writing; AN contribution to writing and data analysis; MH, marker data analysis; RSY, conceptualization, monitoring, review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Rattan S. Yadav https://orcid.org/0000-0001-6132-4541

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Genetic dissection of minerals and phytate content in pearl millet germplasm panel using genomewide association study

Singh, Satbeer; Manwaring, Hanna Rose; Naveen, Aavula; Hegarty, Matthew; Yadav, Rattan S.

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