



RESEARCH ARTICLE

Genetic variation and diversity for grain iron, zinc, protein and agronomic traits in advanced breeding lines of pearl millet [*Pennisetum glaucum* (L.) R. Br.] for biofortification breeding

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Abstract Genetic improvements of iron (Fe) and zinc (Zn) content in pearl millet [*Pennisetum glaucum* (L.) R. Br.] may reduce the problems of anemia and stunted growth among millet dependent staple food consumers. The availability of variation in diverse-breeding lines is essential to improve grain micronutrients in high-yielding cultivars. This study aimed to determine the extent of variability, heritability and diversity for grain Fe, Zn and protein, along with key agronomic traits, in 281 advanced breeding lines bred at ICRISAT and evaluated across two seasons (environments). A pooled analysis of variance displayed significant variation for all these traits. Highest variability was recorded for Fe (35–116 mg kg⁻¹), Zn (21–80 mg kg⁻¹), and protein (6–18%), and a three-fold variation was observed for panicle length, panicle girth and 1000-grain-weight (TGW). Diversity analysis showed 10 clusters. Cluster-III had maximum

lines (25%) and Cluster-V showed the highest mean values for Fe, Zn, protein and TGW. These results highlight the success of breeding program that aimed both the maintenance and creation of genetic variability and diversity. A significant positive correlation among Fe, Zn, protein and TGW indicated the potential for simultaneous improvement. Grain yield had a non-significant association with Fe and Zn, while protein showed a negative correlation. These results suggest that significant variability exists in elite-breeding lines, thus highlighting an opportunity to breed for biofortified varieties without compromising on the grain yield. The lines with high Fe, Zn and protein content can be used as hybrid parents and may also help in further genetic investigations.

Keywords Variability · Correlation · Diversity · Grain iron and zinc · Grain protein · Seed parent · Restorer parent

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Introduction

Pearl millet (*Pennisetum glaucum* (L.) R.Br.) is a climate-smart and nutritionally dependable cereal crop serving several millions of people in sub-Saharan Africa and Asia. Pearl millet is grown in an area of 28 m ha across the world, primarily in the arid and semi-arid regions of Africa (18 m ha) and Asia (> 10 m ha). India has the largest area (7.4 m ha),

with the highest annual production of 9.7 m t (MAFQ, Govt. of India 2017). About 2 billion people across the world suffer from micronutrient deficiencies. While India is the largest producer of pearl millet, recent official data indicate that 59% of children under the age of 5 and 54% of pregnant women are anemic and 38% of children of the same age group are stunted (NFHS-4 2015–2016). Diets deficient in iron (Fe) and zinc (Zn), which are important micronutrients, are the major cause of anemia and stunting, respectively. Pearl millet, being naturally gifted with relatively higher proportions of grain iron (Fe) and zinc (Zn) compared to all other staple cereal food crops, is considered as a potential crop to combat micronutrient malnutrition, especially in the millet growing regions of India (Rajasthan, Gujarat, parts of Haryana, parts of Maharashtra) and sub-Saharan Africa.

At present, the commercially cultivated pearl millet varieties and hybrids have on an average 42 mg kg⁻¹ of Fe and 32 mg kg⁻¹ Zn in the grains (Rai et al. 2016). However, a much wider variability for these micronutrients has been reported in germplasm collections (Rai et al. 2014). Apart from this, hybrids and OPV (Dhanashakti) that have been bred exclusively for Fe and Zn along with grain yield popularly known as biofortified hybrids or varieties which have been released in recent past contains 70–75 mg kg⁻¹ Fe and 35–45 mg kg⁻¹ Zn. These higher levels for Fe and Zn micronutrients should be brought into the current breeding lines and hybrid parents, to achieve adequate nutrition in the end products either in the form of hybrids or OPVs. In spite of recent achievements with respect to improvements in grain productivity (> 188%) in pearl millet (Yadav et al. 2019), a continuous decrease in area and increased demand for nutritionally enhanced grains (Fe and Zn) brings a lot of breeding challenges around maintaining the higher yield potential with adequate nutrition in breeding pipelines. Hence, breeding must concentrate on the simultaneous improvement of grain yield along with the grain quality traits. Apart from grain micronutrients, pearl millet is also a potential source of protein. As protein forms an integral part of the daily nutrient requirements of human beings, improved pearl millet cultivars for protein could possibly help to meet the daily protein requirements of people in pearl millet growing regions. To improve these traits, information on genetic variability, heritability of the trait and diversity in the genetic materials is needed. Further,

complex traits such as grain yield are governed by a large number of minor genes, which are highly influenced by the environment. This necessitates a comprehensive study on the extent of variability owing to the actual heritable genetic variation that is present in the inbreds, which would further help to realize genetic gains for the trait of interest through selection and hence could be exploited through breeding programs. Assessment of correlation between grain yield and micronutrients would further help to decide the type of breeding method to be followed for the improvement of both traits. A considerable amount of variability for grain yield and related traits has been reported in pearl millet using germplasm collections and commercially cultivated hybrids (Kanatti et al. 2014a, b; Rai et al. 2013).

Many breeding lines, belonging to two contrasting heterotic pools (seed parents and restorer parents), are being continuously developed at ICRISAT and other public and private sector organizations. However, very little information exists on the magnitude of variability for nutritional traits and diversity among these advanced breeding lines. Therefore, the current study is aimed at studying the range of genetic variability and the extent of diversity that has been brought into the advanced breeding lines, which include seed-parents, restorers, inbreds derived from germplasms and breeding populations. The associations among grain yield traits, as well as the associations between grain yield and nutritional traits, were also studied.

Material and methods

Genetic materials

The study materials included 281 advanced breeding lines (also referred to as inbreds) with a high-iron variety as a check (Dhanashakti). All lines were expected to have adequate diversity with respect to agronomic traits such as plant height, tillering, panicle size, 1000-grain weight, and grain yield, and were developed at ICRISAT, Patancheru, India. Among these 281 inbred lines, 112 belonged to restorer parents (R-lines) and 110 inbreds belonged to seed parent (B-lines); 32 progenies were derived from breeding population/composites and 27 inbreds were derived from germplasm accessions. The restorations

and maintainer ability of two of the lines were not known at the time of investigation.

Field trials

All the lines and a check were planted during the 2017 rainy season (July to October), and same set of trial was planted during the 2018 summer season (February to May) at ICRISAT, Patancheru. The average rainfall was 207 mm during the rainy crop season and 12.3 mm during the summer crop season. The average minimum and maximum temperatures during the rainy crop season were 23 °C and 31 °C, respectively, whereas 20 °C and 36 °C were the recorded minimum and maximum temperatures, respectively, during the summer crop season. The average relative humidity was higher (77%) in the rainy crop season than the summer (49%). In both seasons, trials were planted in an alpha-lattice design with three replications. Planting was completed using a tractor-mounted 4-cone planter (7100 US model). Each entry was planted continuously in two rows of 2 m long having about 26 plants per plot, with 75 cm between rows in the rainy season and 60 cm in summer season. Overplanted plots were thinned 15 days after planting to single plants spaced 15 cm apart within each row. A basal dose of 100 kg of diammonium phosphate (18% N and 46% P) was applied at the time of field preparation and 100 kg of urea (46% N) was applied as top dressing within 2 to 4 days after thinning. Fields were irrigated at 7 to 10 days intervals in the summer season to avoid any possible moisture stress. All the recommended agronomic practices were followed for good crop growth. At the time of planting, four well spread representative soil samples were collected from the experimental fields from 0 to 30 cm depth and bulked to prepare one composite sample for micronutrient analysis (Lindsay and Norwel 1978). The experimental fields had adequate soil fertility, including the available soil Fe and Zn. Available soil Fe varied from 3.8 to 5.0 mg kg⁻¹ and Zn varied from 1.6 to 2.0 mg kg⁻¹, which was above the critical limits required for normal growth and development (Sahrawat et al. 2002).

The plots of all the entries were harvested at or after physiological maturity (85–90 days after planting) and sundried (2–3 h/day) for 10 to 15 days. The observations were recorded for five representative plants in each plot for plant height (PH), panicle length

(PL) and panicle girth (PG), while days to fifty percent flowering (DFF), thousand grain weight (TGW) and grain yield (GY) were measured on a plot-basis. GY was later expressed in t ha⁻¹. The grain iron (Fe), zinc (Zn) and protein were estimated as follows.

Micronutrient and protein analyses

Grain Fe and Zn densities were analyzed in an Energy-Dispersive X-Ray Fluorescence Spectrometry machine (ED-XRF), model *X-Supreme 8000* from OXFORD, installed in the Pearl Millet Breeding program at ICRISAT, Patancheru. ED-XRF method for pearl millet established reported high correlation between ICP-OES and ED-XRF values $r = > 0.90$ for both Fe and Zn (Govindaraj et al. 2016). The quantified grain iron and zinc levels were measured in milligrams per kilogram (mg kg⁻¹) of seed. The grain protein content was analyzed using near-infrared spectroscopy (NIRS), at ICRISAT, Patancheru. The quantified grain protein (Igne et al. 2007) content was measured in percentage (%).

Statistical analysis

Pooled ANOVA over the 2017 rainy season and 2018 summer season (hereafter referred to as environments) were performed using Generalized Linear Model procedures following a random-effects model (Steel and Torrie 1980; Hallauer and Miranda 1981) in SAS University Edition (SAS/STAT®, SAS Institute Inc, NC, USA) (SAS Inst. 2004). The Pearson's correlation coefficients among the traits were calculated using the PROC CORR procedure in the SAS program.

Associations among the traits were also determined by principal component analysis (PCA) (Hatcher 1994) using R version 3.5.1 (R Project for Statistical Computing, (<https://www.r-project.org>)). These principal components were extracted from the correlation matrix. Data was standardized to estimate the genetic distance matrix using the Euclidean approach distance, then hierarchical clustering was performed using the Ward's distance method (Murtagh and Pierre 2011). The cluster analysis among 281 inbred lines was determined using the statistical package “dendextend” and “circlize” for R version 3.5.1. The number of clusters was fixed at 92% similarity. The significance between the clusters was tested using

Student-Newman-Keul's test. Heritability was determined according to the method of Hanson et al 1956.

$$h^2_{(bs)} = \frac{\sigma_g^2}{(\sigma_g^2 + \frac{\sigma_{gs}^2}{s} + \frac{\sigma_e^2}{rs})}$$

where σ_g^2 is the genotypic variance, σ_{gs}^2 is the genotype \times season interaction variance and σ_e^2 is the residual variance; 'r' is the number of replications and 's' is the number of seasons.

Results

Genetic variance components

The analysis of variance revealed highly significant ($p < 0.01$) differences among inbred lines for grain Fe, Zn, and protein, and for all agronomic traits (Table 1), indicating that there exists a wide range of variability for the traits studied. Environment (E) and Genotype \times Environment (G \times E) interactions showed a highly significant effect on all the traits studied. This significant influence of the G \times E interaction suggested that the genotypes exhibited a differential response to different environments for all the traits studied. Partitioning the sum of squares into different components, such as variation contributed by inbreds alone and variation contributed by the interaction of inbreds with the environment, revealed that the inbreds alone contributed a significantly higher proportion to the traits like grain Fe (92%), Zn (90%),

DFF (81%), PH (93%), PL (95%), PG (96%) and TGW (87%), but not the protein and grain yield, where the contribution of inbreds to these traits was relatively moderate (61% and 62%, respectively).

Inbred performance

The performance of inbreds for Fe varied from 35 mg kg⁻¹ to 116 mg kg⁻¹, with an average grain Fe density of 75 mg kg⁻¹, whereas Zn varied from 21 mg kg⁻¹ to 80 mg kg⁻¹ with an average grain Zn density of 46 mg kg⁻¹ (Table 2). This demonstrated the three to four fold variability for grain Fe and Zn density among these advanced breeding lines (Table 2). Similarly, grain protein content varied from 6 to 18%, with an average grain protein content of 11%. Agronomic traits such as plant height varied from very dwarf (80 cm) to tall (193 cm), with an average plant height of 136 cm; panicle length varied from small (13 cm) to long (36 cm), with an average panicle length of 19 cm; panicle girth varied from medium (15 mm) to thick (40 mm), with average of 28 mm; thousand grain weight varied from 6 to 16 g. Grain yield in these lines varied from 1.3 to 2.4 t ha⁻¹, with an average grain yield of 1.7 t ha⁻¹, which shows the good yield potential that could be achieved by using them as hybrid parents. The results indicate adequate variability and good performance in terms of both agronomic and grain quality traits. For instance, 55% of inbreds have higher Fe, 47% of inbreds have higher Zn and 62% of inbreds have higher protein, exceeding the overall trial mean for the same traits.

Table 1 Analysis of variance for Fe, Zn, protein and agronomic traits over two contrasting environments (rainy-2017 and summer-2018)

Source of variation	d.f	Mean square (MS)									
		Fe	Zn	Protein	DFF	PH	PL	PG	TGW	GY	
Environment (E)	1	96,150.5**	23,085.9**	2587.4**	6721.5**	79,182.9**	328.3**	3.6	55.8**	231.8**	
Replication (R)	4	768.7	377.8**	23.9**	3.0**	228.3**	10.1	7.8**	3.6**	0.3**	
Block (R \times E)	114	43.5	28.3**	74.4**	0.8	50.2**	2.4**	3.6**	0.6	0.1**	
Genotype (G)	280	2075.6**	545.3**	2629.0**	73.9**	2804.3**	58.5**	111.4**	24.6**	0.7**	
G \times E	280	166.0**	55.5**	1024.7**	14.0**	195.2**	3.1**	4.2**	3.2**	0.3**	
Error	1006	37.0	18.5	411.4	0.8	30.5	0.8	1.5	0.6	0.1	

*, **Significant at 0.05% and 0.01% probability, respectively

Fe, Iron; Zn, Zinc; DFF, Days to 50% flowering; PH, Plant height; PL, Panicle length; PG, Panicle girth; TGW, 1000 grain weight; GY, Grain yield

Table 2 Genetic variability for Fe, Zn, protein and agronomic traits over two contrasting environments (rainy-2017 and summer-2018)

Trait	Range		Mean	Heritability (h_{bs}) (%)	CV	SEm
	Min	Max				
Fe (mg kg^{-1})	35	116	75	92.69	8.24	2.72
Zn (mg kg^{-1})	21	80	46	90.14	9.45	1.39
Protein (%)	6	18	11	96.00	5.65	3.06
DFF (d)	46	62	54	81.90	1.59	0.51
PH (cm)	80	193	136	92.76	4.06	3.16
PL (cm)	13	36	19	94.98	4.85	0.46
PG (mm)	15	40	28	96.30	4.33	0.63
TGW (g)	6	16	10	87.50	7.47	0.30
GY (t ha^{-1})	1.3	2.4	1.7	64.10	14.96	0.05

CV, Coefficient of variation; SE, standard error; Fe, Iron; Zn, Zinc; DFF, Days to 50% flowering; PH, Plant height; PL, Panicle length; PG, Panicle girth; TGW, 1000 grain weight; GY, Grain yield

Similarly, with respect to agronomic traits, 48% of inbreds flowered earlier, 52% of inbreds were taller, 34% had longer panicles and 53% recorded thicker panicles when compared to the overall trial mean. More than 43% of inbreds showed a higher TGW and higher GY over the trial mean. Assessing the performances of the top 10% of high-Fe inbreds (28) (Table 3), with $\geq 100 \text{ mg kg}^{-1}$ Fe compared with the low-Fe ($\leq 43 \text{ mg kg}^{-1}$) group of inbreds, it was found that the high-Fe group had an 18% higher mean for grain protein (Supplementary Table 1). Both the groups had similar mean values with respect to GY, DFF and PH, while the high-Fe group had significantly higher mean values for TGW (32%) and PG (22%), and significantly lower mean values for PL (21%). High broad sense heritability ($> 90\%$) was observed for PG, protein, PL, PH, Fe and Zn.

Traits association among Fe, Zn, protein and agronomic traits

A significant positive association was observed between grain Fe and Zn ($r = 0.76$; $p < 0.01$) (Fig. 1). Furthermore, both grain Fe and Zn showed significant positive associations with protein, PH, PG and TGW. A significant but negative association was observed for PL ($p < 0.01$), and a negative but non-significant association was observed for DFF. Grain Fe showed a significant positive association with GY ($r = 0.14$; $p < 0.05$), while grain Zn showed a

negative but non-significant association with GY ($r = -0.01$). Likewise, the grain protein content showed significant but negative associations with PL ($r = -0.32$; $p < 0.01$), GY ($r = -0.29$; $p < 0.01$) and DFF ($r = -0.26$; $p < 0.01$). Interestingly, grain protein showed a significant positive association with TGW ($r = 0.15$; $p < 0.01$). Grain yield showed a significant negative association with DFF ($r = -0.19$; $p < 0.01$), and significant positive associations with PG ($r = 0.48$; $p < 0.01$) and TGW ($r = 0.48$; $p < 0.01$).

Principal component analysis

A total of nine principal components (PCs) were calculated (Table 4). The eigen values for the first three PCs were 2.89, 1.78 and 1.52 for PC1, PC2 and PC3, respectively. These three principal components together contributed 68.75% to the total variation. Among the first three principal components, grain Fe (0.49), Zn (0.43) and TGW (0.44) contributed more towards PC1 (Table 5). GY (0.58) and protein (0.50) contributed the most towards PC2, whereas PH (0.57) and DFF (0.56) contributed more towards PC3 (Table 5). Following the principle in Rad et al 2013 work, the present study revealed that the contributions of grain Fe, Zn and protein were in the positive direction, towards PC1 forming a single group, as the angles between them were less than 90° (Fig. 2a, b). Similarly, PG, TGW and GY contributed more in the positive direction towards PC2 and together they

Table 3 Selected top 28 (10%) high Fe inbred performances for Zn, protein and agronomic traits over two contrasting environments (rainy-2017 and summer-2018)

Line designation	Fe (mg kg ⁻¹)	Zn(mg kg ⁻¹)	Protein (%)	DFF (d)	PH (cm)	PL (cm)	PG (mm)	TGW (g)	GY (t ha ⁻¹)
ICMB 100648	120	71	13	54	145.38	17.68	27.54	13.51	1.39
ICMR 1502	117	64	12	54	141.96	17.58	28.25	11.88	1.50
ICMP 100429	116	71	13	44	130.29	15.97	26.16	13.22	1.59
ICMR 100978	115	87	14	53	138.97	13.87	28.76	11.16	1.24
ICMR 100236	114	73	14	51	130.93	17.66	26.46	7.33	1.39
ICMR 100102	114	56	13	56	149.22	19.15	35.78	12.97	1.20
ICMP 100410	113	54	12	53	173.54	16.12	26.62	12.69	1.62
ICMB 100680	113	65	13	49	122.13	15.30	28.11	15.30	1.42
ICMP 100421	112	51	13	52	133.93	13.43	27.36	11.59	1.44
ICMP 100436	112	63	15	55	172.29	20.03	20.36	11.26	1.20
ICMB 100617	110	54	12	52	146.17	17.83	26.33	13.14	1.64
ICMR 100152	110	59	12	63	161.50	20.47	32.90	10.26	1.35
ICMB 100454	109	53	14	46	125.50	13.67	28.32	15.95	1.54
ICMP 100433	109	59	12	46	137.25	17.47	28.03	13.26	1.87
ICMR 100725	106	59	14	52	104.89	13.48	27.27	8.25	1.13
ICMB 100664	106	58	13	46	115.73	16.90	27.06	16.44	1.84
ICMR 100142	105	48	13	54	154.83	19.87	31.87	10.66	1.82
ICMR 100071	104	57	11	59	138.23	16.87	23.99	8.08	1.16
ICMB 100645	104	55	11	53	150.20	15.56	28.61	13.38	1.74
ICMB 100667	104	57	12	57	128.75	19.84	34.84	11.11	1.45
ICMB 100631	102	49	10	57	137.60	18.93	29.61	13.16	1.87
ICMB 100668	102	53	12	47	110.83	13.33	31.15	12.27	2.07
ICMR 101008	102	47	13	62	156.87	21.40	30.42	9.35	1.91
ICMR 101010	102	57	12	55	138.23	19.07	28.16	10.44	1.79
ICMP 100424	101	61	12	56	161.70	15.34	28.48	11.19	1.22
ICMP 100434	101	68	14	48	154.96	14.38	25.81	10.34	1.62
ICMR 100109	100	50	10	57	165.50	16.47	32.03	10.52	2.11
ICMR 100998	100	54	12	58	171.23	16.90	31.07	9.92	1.81

Fe, Iron; Zn, Zinc; DFF, Days to 50% flowering; PH, Plant height; PL, Panicle length; PG, Panicle girth; TGW, 1000 grain weight; GY, Grain yield; ICMB, ICRISAT Millet B–line; ICMR, ICRISAT Millet Restorer; ICMP, ICRISAT Millet Population progenies

formed another group. DFF, PH and PL contributed more in a positive direction towards PC3, forming the third group.

Diversity analysis and cluster means

The phenotypic diversity analyses partitioned the 281 inbred lines into 10 different clusters at 92% similarity

(Fig. 3). Cluster III was the largest, comprising 70 inbreds including 26 R-lines and 40 B-lines, followed by cluster VII with 48 inbreds, including 27 R-lines and 3 B-lines, while cluster VIII had 33 (15 R-lines and 2 B-lines) inbreds. Cluster X had the least number of entries (3 inbreds, Table 6). Cluster V (4 R-line and 10 B-lines) showed the highest cluster mean for grain Fe density (102 mg kg⁻¹), with higher variation

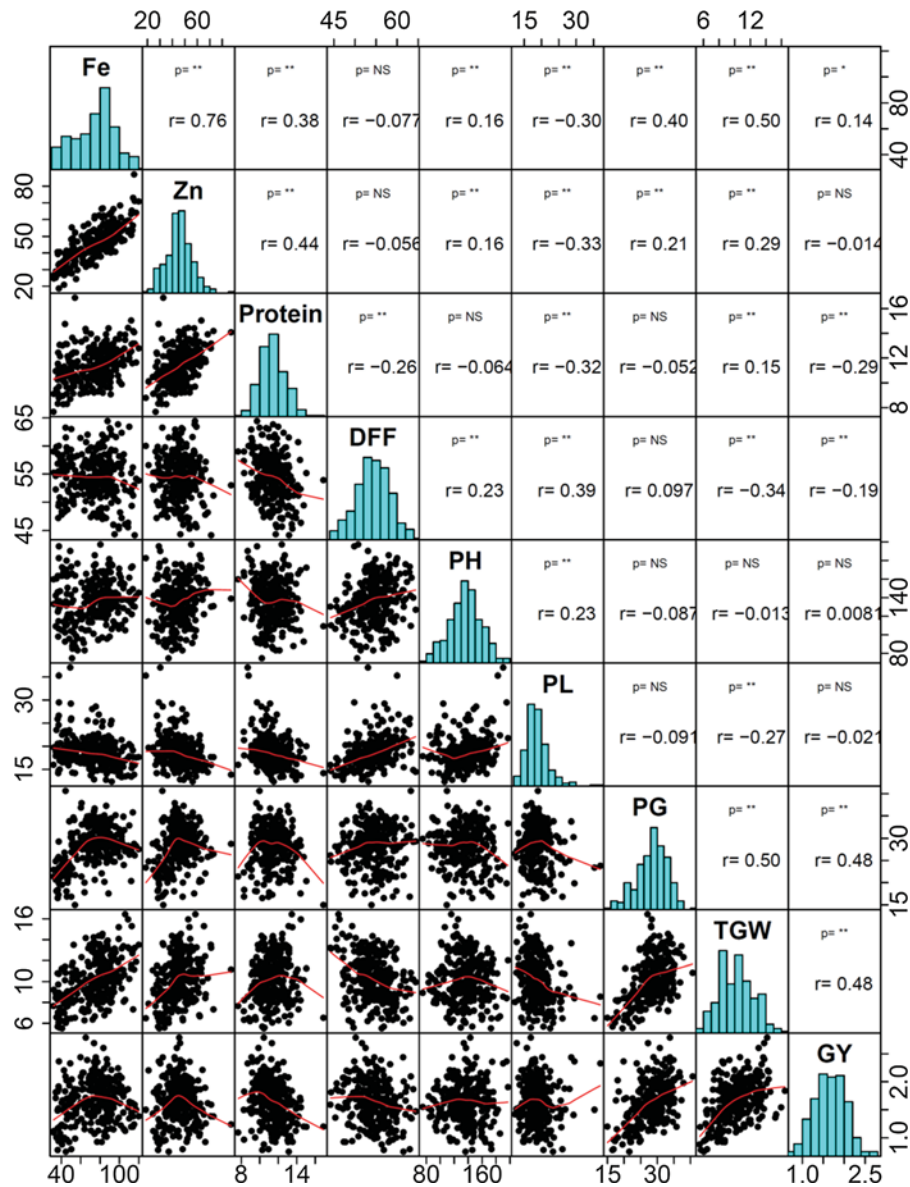


Fig. 1 Phenotypic correlation and frequency distribution for grain Fe, Zn densities, Protein and Agronomic traits

accumulated in these inbreds ($82\text{--}116 \text{ mg kg}^{-1}$). Furthermore, cluster V also recorded higher cluster means for Zn, protein, early-flowering and TGW. Cluster X (2 R-lines) showed the lowest (41 mg kg^{-1}) mean values for Fe, Zn and protein, whereas it showed the highest cluster mean for grain yield. Cluster IX (1 R-line) showed the highest cluster mean for protein (14%), ranging from 13 to 15% and also recorded a higher cluster mean for Zn but had the lowest grain yield potential (Table 6). Cluster II captured late-flowering lines (58 days).

Discussion

The availability of adequate variability in the working germplasm of grain quality traits is a prerequisite to begin biofortification breeding to improve grain nutrition. The present study evidenced that the significant mean squares for all the traits displayed substantial variability among the 281 inbreds for grain Fe, Zn, protein and agronomic traits. The influence of $G \times E$ interactions is challenging for any trait breeding. The results of this study indicated the presence of a

Table 4 Eigen value and percent variance contribution of each PCA components

PCA	Eigen value	Variance (%)	Cumulative. variance (%)
PC1	2.89	32.1	32.1
PC2	1.78	19.77	51.87
PC3	1.52	16.88	68.75
PC4	0.89	9.89	78.64
PC5	0.61	6.81	85.45
PC6	0.45	5.03	90.48
PC7	0.37	4.09	94.57
PC8	0.3	3.3	97.87
PC9	0.19	2.13	100

Table 5 PCA loadings corresponding to each level for Fe, Zn, protein and agronomic traits evaluated over two contrasting environments (rainy-2017 and summer-2018)

PCA Loadings for each trait									
Traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Fe	0.49	- 0.07	0.31	- 0.05	0.02	0.26	- 0.19	- 0.05	- 0.74
Zn	0.43	- 0.24	0.33	- 0.06	0.15	0.50	0.07	- 0.01	0.61
Protein	0.28	- 0.50	- 0.01	- 0.05	- 0.54	- 0.33	0.46	0.22	- 0.03
DFF	- 0.22	0.12	0.56	- 0.50	0.16	- 0.22	- 0.01	0.54	0.00
PH	- 0.02	0.03	0.57	0.67	0.15	- 0.38	0.12	- 0.18	0.03
PL	- 0.33	0.24	0.33	0.07	- 0.75	0.37	- 0.08	- 0.10	0.03
PG	0.32	0.45	0.10	- 0.44	- 0.09	- 0.32	0.20	- 0.58	0.11
TGW	0.44	0.27	- 0.11	0.16	- 0.25	- 0.29	- 0.61	0.34	0.24
GY	0.21	0.58	- 0.14	0.23	0.05	0.24	0.56	0.41	- 0.08

Fe, Iron; Zn, Zinc; DFF, Days to 50% flowering; PH, Plant height; PL, Panicle length; PG, Panicle girth; TGW, 1000 grain weight; GY, Grain yield

G × E effect for Fe, Zn, protein and the expression of the agronomic traits studied. However, the G × E magnitude, particularly for Fe, Zn and agronomic traits, was negligible, as the variation contributed by the genotype (inbreds) was much higher (> 80%) than the contribution of G × E. This suggested that selection based on performance would be rewarding for Fe and Zn, along with other agronomic traits. The contributions of the inbreds to protein and grain yield were moderate, but higher than the G × E component. A similar low contribution of G × E has been reported for grain Fe and Zn density in pearl millet (Govindaraj et al. 2015; Kanatti et al. 2014a; Anuradha et al. 2017), and a higher G × E for protein and grain yield (Gebre 2014), in pearl millet. Since the differential uptake and partitioning is largely a genotypic dependent process, adequate and more availability of soil micronutrients (Fe and Zn) has not been expected influenced accelerated accumulation to grains. Previous study stated the non-significant relationship between soil Fe/Zn

and grain Fe/Zn in pearl millet (Govindaraj et al. 2019).

Heritability is the inherent capacity of the genotype, monitored during selection and used as an indicator of trait transmission from one generation to the next, or from parents to progeny. High heritability was observed for grain Fe, Zn and protein, along with all the agronomic traits studied, which suggests that simple selection and advancement would increase the genetic gain of these traits. Further, for most of these traits (including Fe and Zn) the high heritability was due to additive gene control therefore, high heritability traits are likely to respond to direct selection. Similar high heritability for Fe, Zn and protein have also been reported previously in pearl millet (Vagadiya et al. 2013; Vinodhana et al. 2013; Kumar et al. 2014).

A highly significant and positive correlation was found between grain Fe and Zn. This finding is in accordance with the previous studies on pearl millet (Rai et al. 2012, 2014; Velu et al. 2007, 2008; Gupta

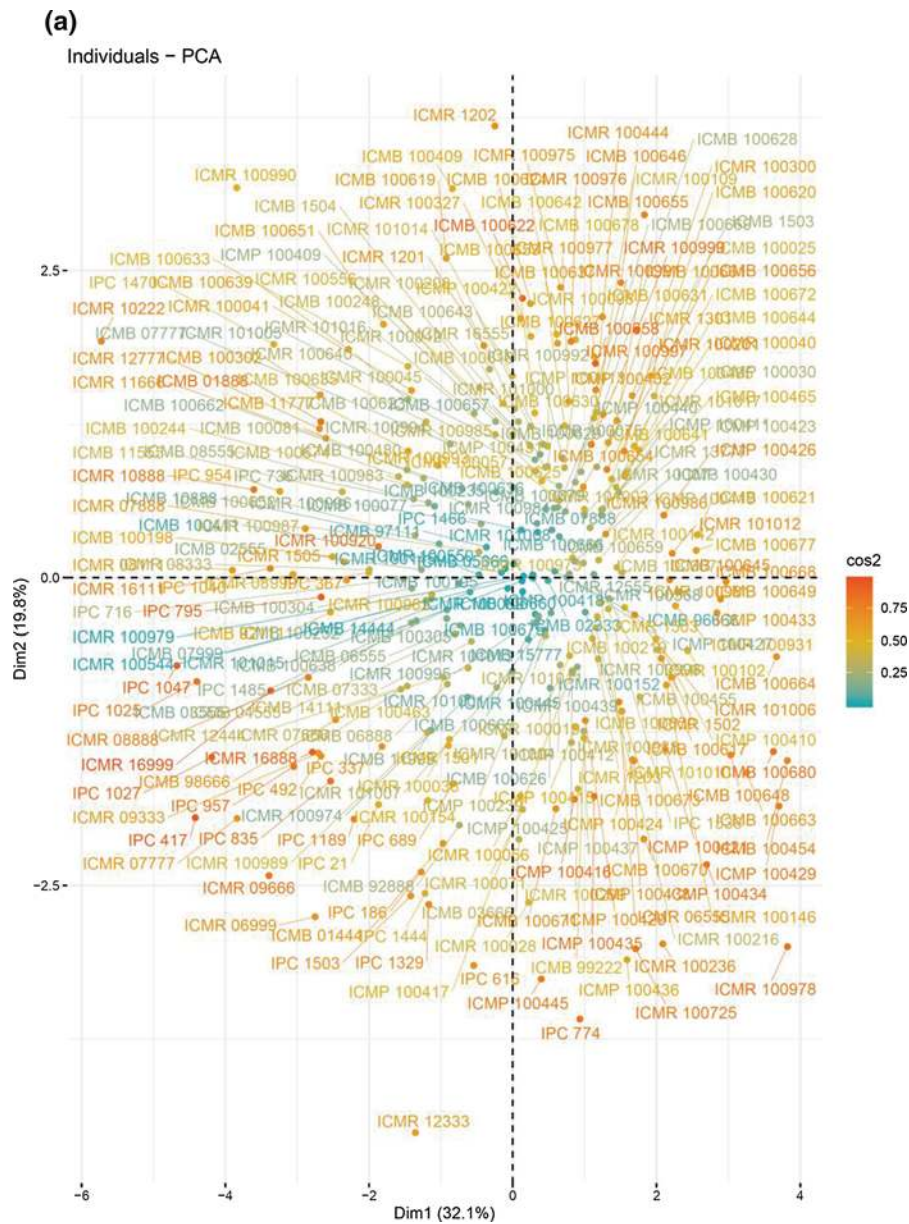


Fig. 2 a. Principal component analysis (PCA) for individuals (inbreds). **b.** Principal component analysis (PCA) for nine variables (traits), grain iron (Fe) and zinc (Zn) densities,

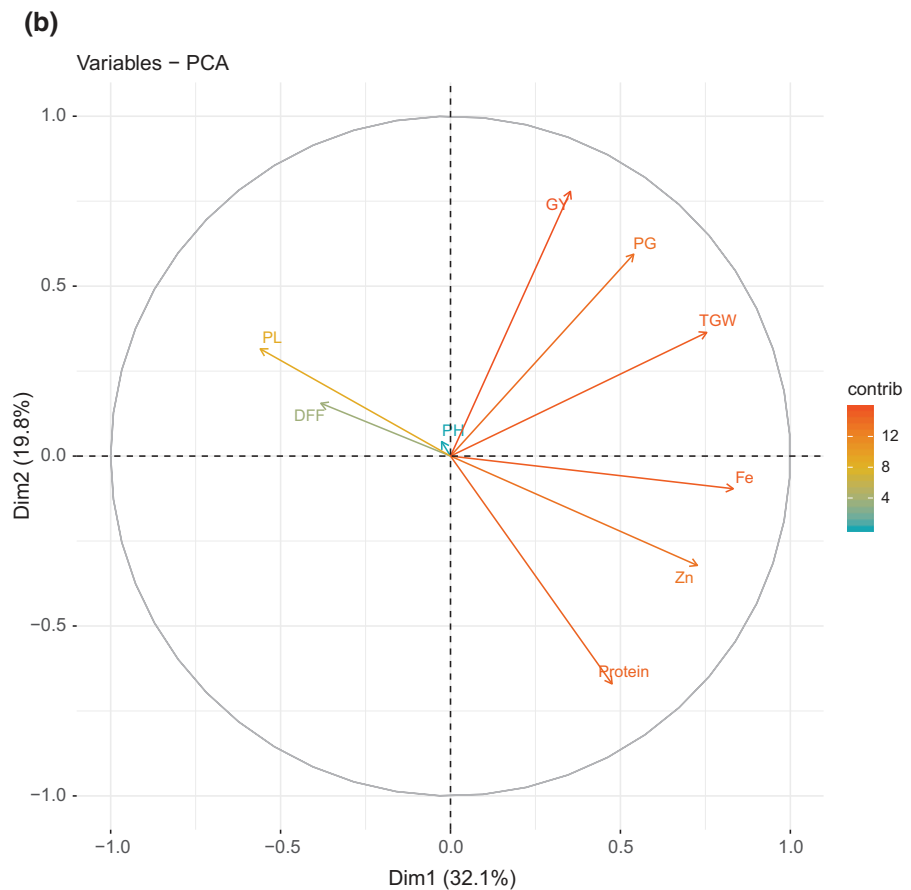
1000-grain weight (TGW), grain yield (GY), plant height (PH), panicle length (PL) and panicle girth (PG)

et al. 2009; Govindaraj et al. 2013). These positive associations between Fe and Zn densities would have resulted from a common physiological mechanism or co-localized quantitative trait loci (QTLs) for Fe and Zn accumulation, as reported in pearl millet (Kumar 2011), wheat (Peleg et al. 2008) and rice (Stangoulis et al. 2007), implying that simultaneous selection for both micronutrients could be effective. This consistent

association across environments provides opportunities to rank genotypes for Fe and Zn, and these can be expected to be less influenced by environments.

Both Fe and Zn showed significant and positive associations with grain protein, indicating the possibility of improvement of all three nutrient traits simultaneously. In other words, improvement for grain iron and zinc would not affect or reduce the protein

Fig. 2 continued



content in the grains. Both grain Fe and Zn densities showed highly significant positive associations with TGW. A similar trend was noticed among the top 20% of the high Fe lines. This substantiates that the TGW has a significantly positive linkage with Fe/Zn selection and improvement. Grain Fe showed positive significant and grain Zn showed negative non-significant associations with grain yield. Further, the correlation of the top 20% high Fe lines was significant and negative for Fe/Zn and GY. The bottom 20% of the low Fe inbreds displayed non-significant positive associations between Fe/Zn with GY. This is expected since these lines were selected and advanced primarily for agronomic traits and not targeted for grain quality aspects. This negative linkage can be broken by conscious selection in larger segregating populations of crosses involving high-Fe and high-yielding parents. Interestingly, in the top 20% of the high Fe group, 43% of the inbreds showed a higher grain yield than the overall trial mean. These results support the fact

that the selection for high Fe and Zn in pearl millet is highly possible without compromising on grain yield potential. Since both the micronutrients are additively controlled, incorporation of these traits into both male and female is mandatory to get high Fe/Zn and yielding hybrids. Like Fe and Zn, grain protein also showed a significant positive association with TGW. Further, associations among the top 20% of high protein lines showed lower magnitudes, but significantly negative associations with grain yield, indicating the practical difficulty of improving the protein and grain yield simultaneously. Previous studies in pearl millet (Sharma et al. 2018), and wheat (Moore et al. 2015; Singh and Kumar 2017; Rathod et al. 2019; Spika et al. 2019) have reported similar negative association between protein and GY.

Principal component analysis gives us information regarding the importance and contribution of each component to the total variance (Sanni et al. 2012). The first three PCs contributed the most variability

Inbred groups
 R-line (Restorer parent)
 B-line (Seed parent)
 ICMP (Population progeny)
 IPC (Germplasm progeny)

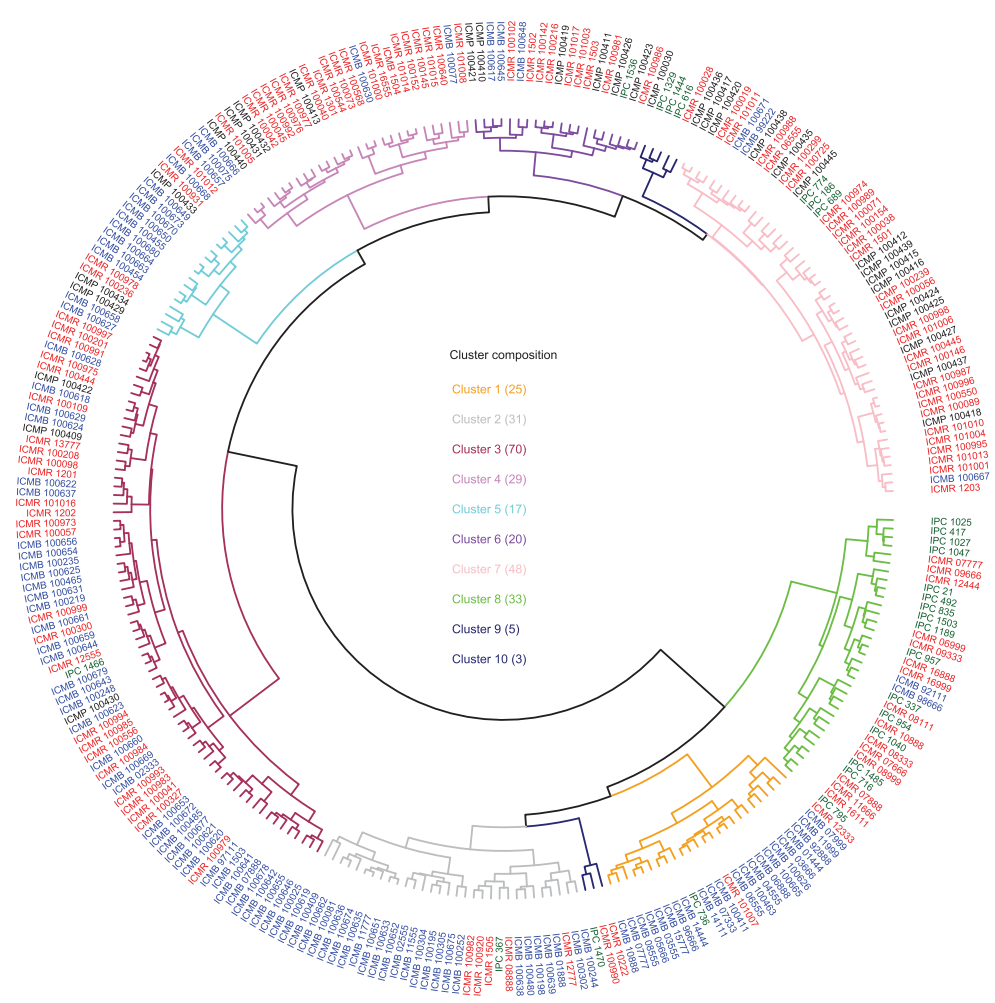


Fig. 3 Clustering pattern (pooled over rainy-2017 and summer-2018 seasons, ICRISAT, Patancheru) of the 281 pearl millet inbred lines

(68.75%) to the total variance. Grain Fe and Zn, as well as TGW, contributed most to the diversity captured in the first principal component (PC1), which were in the positive direction. Protein (negative) and PG (positive) contributed most to the diversity

captured in the second principal component (PC2), whereas DFF and PH contributed most to the diversity captured in the third principal component (PC3) in the positive direction. Cluster analysis among 281 inbred lines showing significant variability for grain Fe, Zn,

Table 6 Cluster means for Fe, Zn, protein and agronomic traits among 281 inbreds evaluated over two contrasting environments (rainy-2017 and summer-2018)

Clusters	No. of genotypes	Fe (mg kg ⁻¹)	Zn (mg kg ⁻¹)	Protein (%)	DFP (d)	PH (cm)	PL (cm)	PG (mm)	TGW (g)	GY (t ha ⁻¹)
Cluster-I	25	56 ^c	40 ^{de}	12 ^b ^c	52 ^c	104 ^f	18 ^{cd}	26 ^c	9.8 ^{bc}	1.57 ^{bc}
Cluster-II	31	60 ^c	41 ^{de}	11 ^d	58 ^{ab}	117 ^{ef}	23 ^b	29 ^{abc}	8.7 ^c	1.66 ^b
Cluster-III	70	77 ^b	44 ^{cd}	11 ^{cd}	53 ^c	137 ^{cd}	18 ^{cd}	31 ^{ab}	11.4 ^{ab}	2.02 ^a
Cluster-IV	29	86 ^{ab}	47 ^{bcd}	11 ^{cd}	60 ^a	146 ^{cd}	20 ^{bc}	32 ^a	11.2 ^{ab}	1.7 ^b
Cluster-V	17	102 ^a	59 ^a	13 ^{ab}	48 ^d	124 ^{de}	15 ^d	27 ^{bc}	12.7 ^a	1.69 ^b
Cluster-VI	20	97 ^{ab}	55 ^{ab}	12 ^{bc}	53 ^{bc}	154 ^c	18 ^{cd}	30 ^{abc}	12.2 ^a	1.7 ^b
Cluster-VII	48	87 ^{ab}	53 ^{abc}	12 ^{bc}	55	143 ^{cd}	18 ^{cd}	28 ^{abc}	9.6 ^{bc}	1.4 ^{bcd}
Cluster-VIII	33	42 ^c	33 ^{ef}	11 ^d	55 ^{bc}	144 ^{cd}	19 ^c	21 ^d	7.6 ^c	1.26 ^{cd}
Cluster-IX	5	83 ^{ab}	59 ^a	14 ^a	55 ^{bc}	170 ^b	21 ^{bc}	20 ^d	8.2 ^c	1.19 ^d
Cluster-X	3	41 ^c	26 ^f	9 ^e	54 ^{bc}	189 ^a	34 ^a	22 ^d	7.8 ^c	2.06 ^a

Superscripts alphabets represents the significance differences between the clusters (within column)

protein and agronomic traits were grouped into 10 distinguished clusters at 92% similarity and were significantly different from each other. Critical examination of individual clusters revealed that the majority of the high Fe lines were well distributed among six clusters (Cluster III, IV, V, VI, VII and IX), among which cluster V contained all high Fe lines. Furthermore, all six of these clusters with high Fe lines were found to be highly diverse with respect to agronomic performance.

Hybrid-parents breeding in pearl millet deals with two heterotic groups, known as seed parents (B-line) and restorer parents (R-line) (Rai et al. 2014). Breeding for restorer parents (R-line) will target profuse pollen production, taller plant height and a greater number of tillers. Dwarf plant types and large grain size, on the other hand, were taken into account while selecting/breeding for seed parents (B-lines). In the present study, cluster I, II and V together had the maximum number of seed parents and were distinguished from others with a few exceptions. Further, the population progenies (ICMP) and germplasm progenies (IPC) were derived in order to use as both seed parents and restorers, depending on their maintainer and restoration ability. This allele frequency and similarity in pedigree origin may lead to the random grouping of IPC and ICMP among both the seed and

restorer groups. In the past, such progenies were largely used as restorers due to their plant traits and major allele frequency for fertility restorations. This was reflected in our study. Cluster VII, VIII and IV largely had restorers, with a few exceptions of the ICMP and IPC lines. Therefore, progenies of ICMP and IPC are necessary to test for their restoration/maintainer ability before they can be used as potential parents for new crosses in hybrid breeding. The present findings were similar to the previous results found for pearl millet (Anuradha et al. 2017; Chaudhary et al. 2015; Shanmuganathan et al. 2006; Singh et al. 2017; Ramya et al. 2017). Furthermore, these findings suggest that the inbreds used in the present study showed high variability for the traits studied, such as grain Fe, Zn and protein, which belong to different clustering groups and can be used as parents to exploit heterosis for grain yield and nutritional traits. There were total of 10 clusters which showed differential cluster means exclusively for grain Fe and Zn. Further, those lines among different clusters showing considerable variations for Fe, Zn, protein and grain yield traits can be further be used for comprehensive studies, such as those on association mapping, genome wide association studies (GWAS), or QTL mapping, in order to identify the molecular markers that would further fasten the process of

biofortification for Fe, Zn and protein. This would help considerably in reducing the time taken to develop a biofortified hybrid or an open pollinated variety through a conventional breeding program.

Conclusion

This study reported wide variability for grain micronutrients (Fe and Zn), protein and grain yield traits in advanced breeding lines that are likely to be used as future parents. The high heritability for these traits reported here indicate the minor influence of $G \times E$, showing that simple selection-based breeding will be effective for genetic improvement. Positive and significant correlations among Fe, Zn, protein and TGW imply the possibility of concurrent genetic improvement through conventional breeding. A positive association between grain iron and grain yield suggests that the biofortification for Fe/Zn with competitive yields is possible. Ten distinguished clusters with two-to-four-fold variability for these traits indicated advanced breeding lines bred so far at ICRISAT have significant trait-specific and diversity arrays of materials. Accessibility and use of these elite nutri-dense breeding lines (germplasms) in public and private breeding organizations may benefit nutrition trait introgression and mainstreaming for commercial hybrid breeding in the near future.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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