Identification of simple sequence repeat markers for grain iron and zinc content in pearl millet (*Pennisetum glaucum* (L.) R. Br.)

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Pearl millet is an important staple crop, and biofortifying with grain iron (Fe) and zinc (Zn) is a continuing effort to fight micronutrients malnutrition globally. In the present study, parental polymorphism survey identified 99 polymorphic primers between the contrasting parents out of 275 primers used. Further, in bulk segregant analysis, two simple sequence repeats (SSRs), *Xipes0027* and *Xpsmp2263* were found to be polymorphic between high and low grain Fe and Zn content parents as well as bulks. The primer *Xipes0027* showed the specific band of 214 bp and *Xpsmp2263* amplified 238 bp in the high grain Fe and Zn content parents and bulks. Moreover, individual plants of each contrasting bulks were genotyped to validate the result of BSA. *Xipes0027* was reported to be present on linkage groups 2 as well as 6 of pearl millet, and *Xpsmp2263* was located on linkage group 7 according to the published SSR consensus maps. As a result, these markers have been reported to be putatively linked to elevated Fe and Zn content in grains of pearl millet.

Keywords: Biofortification, bulk segregant analysis, grain Fe and Zn content, pearl millet, SSRs.

PEARL millet (*Pennisetum glaucum* (L.) R. Br.) Syn. *Cenchrus americans* (L.) (Morrone), is botanically a member of the family, Gramineae. The crop is distinctly cross pollinated and diploid ($2n = 2x = 14$) with a genome of 1.79 Gb (ref. 1). Pearl millet is a climate resilient C_4 plant with high efficiency of photosynthesis and biomass producing capacity. In terms of arable area, it is the fifth most significant cereal crop globally and sixth important crop of south Asia[2,3]. It is a prime nutritious cereal with coarse grain cultivated primarily for grain and fodder purpose in the semi-arid tropical environmental conditions of Asia and Africa. With 8.61 million tonnes of pearl millet grains produced from an occupied area of 6.93 million ha and an average productivity of 1243 kg/ha, India is the world's largest grower of pearl millet[2,3]. In the warmest and driest drought-prone regions of Africa and the Indian subcontinent, where rainfed agriculture is prominent, it is a source of income for millions of rural families. Pearl millet is a hardy crop and adapted to extreme environments especially deficient moisture and high temperature. It thrives in hot and dry climates with an annual rainfall as low as 250 mm, where other major cereals are likely to fail[4]. Furthermore, during the reproductive stage, the crop has the ability to withstand air temperatures of more than 42°C. As a result, it is a popular crop in India's western and northwestern summers[5].

In comparison to other cereals, pearl millet is a good source of dietary protein (8–19%), low in starch, high in fibre (1.2 g/100 g), fat, Ca, P, Fe, Zn and essential amino acids[6]. Furthermore, the crop contains enough genetic variations for grain iron (Fe) and zinc (Zn), indicating that it has high potential to combat malnutrition[7,8]. Iron deficiency anaemia is the most common health disorder, affecting mostly low-income nations and resulting in maternal and child fatality as well as other physiological problems[7–9]. Stunting, increased susceptibility to various infectious diseases, morbidity, and low mental ability are all possible consequences of zinc deficiency[7,9,10]. Thus, malnutrition caused by lack of one or other micronutrients is a global health problem that affects over 70% of the world's population[11]. Among all the feasible ways to fight micronutrient deficiencies, genetic biofortification is viable and a cost effective approach[11,12].

Most of the traits for agronomic and grain micronutrients' concentrations are genetically complex in nature, and are controlled by polygenes, environments and interactions among them[5,13–16]. Identification of genes which control the quantitative traits variation plays a major role in understanding the genetic architecture and manipulating the traits of interest accordingly[17,18]. However, conventional methods require phenotyping of all individuals collected from a sample population for the traits targeted. Therefore, it is usually high-priced and time-consuming[19]. To keep the data robust by decreasing the cost incurred and simplifying analytical process, selective analysis technique, such as selective genotyping, where, only individuals from two extremes (typically the two end tails sorted out from a sample population) are analysed, is

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Table 1. Details of female and male parent, pedigree and source/origin

Genotype	Pedigree	Source/origin	Characteristic features (Fe and Zn in ppm)
J 2340	(F-298 × F_4FC 1498)-3-12-2-1-B	PMRS, JAU Jamnagar	Fe = 35.40, Zn = 21.80
30291	((EEBC S_1-407-1-B-B-B-B-B-1-B-1-B-13-1 × B-bulk (3981-3989/S06 G1))-1-2-3 × ((ICMR 312 S1-1-5-2-B × HHVBC)-10-2-1-2-3 × EEBC 407)-7-2-1-3)-11-4-3	ICRISAT, Patancheru	Fe = 102.80, Zn = 45.00

ppm indicates parts per million.

proposed[20]. More recently, bulk segregant analysis (BSA) was modified to tag the minor genes governing quantitative traits, employing large populations, enlarged tail sizes and high density markers eliminating the requirement to validate putative markers by genotyping whole populations with positive markers[20,21]. As a result, by employing selective samples, though it involves substantially low genotyping costs, the statistical power in quantitative trait locus (QTL) mapping is still comparable to the total population analysis[20]. Initially, BSA was designed to tag the traits governed by major genes that were much less affected by environmental interactions[19]. The pooled DNA analysis from two contrasting or extreme groups of individuals from segregating population is used to tag the traits[18,20]. Quantitative trait analysis is usually associated with a mapping population of plants, each of which has to be genotyped with all the markers selected to cover the genome as well as phenotyped for the traits of interest. Genotyping of a large mapping population is tedious and relatively costly. Depending on whether these plants are generated from a cross between two diverse parental lines, or from a population of plants with varied genetic origins, e.g. variety mixtures or composite populations, two variants of the BSA are possible. This was first proposed for use in plant genetics by Michelmore *et al.*[22] who tagged disease resistance by genotyping contrasting bulks with random amplified polymorphic DNAs (RAPDs). They also described identification of markers linked to particular trait using other marker techniques such as restriction fragment length polymorphisms or RAPDs or microsatellite markers through BSA, over other genetic techniques of gene 'tagging', such as use of near isogenic lines (NILs), produced by repeated back crossing which is a tedious and cumbersome process[22]. Furthermore, BSA for polygenic traits showing normal distribution in a population, where samples from two extremes/tails are selected and bulked for genotyping has been proposed.

Materials and methods

Plant materials

Plant materials used in the current study comprised of two contrasting parents – female parent J 2340 was used as a low Fe and Zn content parent, whereas male parent 30291 was used as a high Fe and Zn content parent (Table 1). The parent J 2340 had longer plant height with few tillers, longer panicle length and possessed low Fe (35.40 ppm) and Zn (21.80 ppm) content, whereas the parent 30291 was medium in height with moderate tillers, medium panicle length and contained very high Fe (102.80 ppm) and Zn (45.00 ppm) content. Genotype J 2340 was received from Pearl Millet Research Station (PMRS), Junagadh Agricultural University (JAU), Jamnagar, Gujarat, and genotype 30291 was obtained from pearl millet breeding programme of International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, India.

Development of mapping population

During summer 2016, crosses were made between the two selected contrasting parents to generate F_1 seeds. F_1s were sown at the Centre for Crop Improvement, Sardarkrushinagar in *kharif* 2016, and the whole panicle of F_1 plants was covered with white parchment paper bags to prevent cross pollination to produce F_2 seeds. Selfed seeds of F_1 plants were sown along with their parents to develop F_2 mapping population in *kharif* 2016. During summer 2017, $F_{2:3}$ families were generated from the bagged panicles of 201 individual F_2 plants in plant-to-row pattern.

Grain micronutrients analysis

The $F_{2:3}$ seeds (seeds from individually sown F_2 plants) harvested from 10 competitive plants in 2017 summer along with diverse parents, J 2340 and 30291 were analysed for micronutrients concentration. Each entry's cleaned grains were dried in oven at 60°C for 48 h before being ground to a fine powder with a mortar and pestle. For subsequent analysis, ground samples were appropriately labelled and stored in butter paper covers. The grain micronutrients concentrations were estimated by wet acid digestion procedure from the extract using diacid mixture as suggested by Singh *et al.*[23] at the Centre for Bioresearch Laboratory, S. D. Agricultural University, S. K. Nagar, Gujarat. One gram of finely ground sample was pre-digested with 10 ml concentrated HNO_3 and stored overnight. Further, diacid mixture (HNO_3 and $HClO_4$) was prepared and approximately 10 ml was added to the

Table 2. Mean values showing grain Fe and Zn content of J 2340 × 30291 derived $F_{2:3}$ progenies

Genotype number designated in F_2 generations	Nature of genotype and number designated in BSA	Mean ± SD for Fe content (ppm) in $F_{2:3}$ progenies	Mean ± SD for Zn content (ppm) in $F_{2:3}$ progenies
1	Low Fe and Zn plant 1	38.02 ± 0.73	22.06 ± 0.54
30	Low Fe and Zn plant 2	41.26 ± 0.10	22.44 ± 0.05
51	Low Fe and Zn plant 3	43.35 ± 0.84	23.51 ± 0.78
52	Low Fe and Zn plant 4	44.09 ± 0.22	23.05 ± 0.16
54	Low Fe and Zn plant 5	45.61 ± 0.51	24.09 ± 0.49
56	Low Fe and Zn plant 6	47.24 ± 0.62	24.11 ± 0.57
58	Low Fe and Zn plant 7	48.33 ± 0.09	25.55 ± 0.06
115	Low Fe and Zn plant 8	44.08 ± 0.14	23.41 ± 0.11
116	Low Fe and Zn plant 9	47.24 ± 0.75	25.33 ± 0.67
193	Low Fe and Zn plant 10	46.07 ± 0.64	24.08 ± 0.54
41	High Fe and Zn plant 1	103.32 ± 0.53	47.19 ± 0.44
35	High Fe and Zn plant 2	103.07 ± 0.08	46.30 ± 0.01
76	High Fe and Zn plant 3	102.81 ± 0.65	46.16 ± 0.57
80	High Fe and Zn plant 4	100.55 ± 0.44	45.07 ± 0.32
82	High Fe and Zn plant 5	101.48 ± 0.67	46.77 ± 0.54
83	High Fe and Zn plant 6	98.06 ± 0.39	44.52 ± 0.41
80	High Fe and Zn plant 7	100.11 ± 0.57	45.34 ± 0.43
179	High Fe and Zn plant 8	101.29 ± 0.17	46.14 ± 0.04
182	High Fe and Zn plant 9	99.57 ± 0.39	45.32 ± 0.20
180	High Fe and Zn plant 10	99.42 ± 0.80	44.58 ± 0.74

SD indicates standard deviation.

pre-digested sample. The samples were then placed on a hot plate at 90°C until the fumes emitted were colourless. The digested volume was filtered with Whatman filter paper and the final volume was made up to 100 ml by pouring double distilled water in long neck and round bottom conical flask. Each step was carefully monitored to ensure that samples were not contaminated by extraneous dust particles. The samples were analysed for Fe and Zn density by atomic absorption spectrophotometer (AAS), ELICO SL 194. The mean values of Fe and Zn content of 10 low and 10 high genotypes of $F_{2:3}$ plants are presented in Table 2.

Molecular analysis using SSRs

Genomic DNA was isolated from 10 high and 10 low Fe and Zn content plants of F_2 (based on pooled data on 10 plants of $F_{2:3}$ families) and parental lines using modified cetyl trimethyl ammonium bromide method[24]. The DNA samples were quantified on Eppendorf UV biospectrophotometer. Two microlitres of DNA was held in cuvette through micropipette for measurement of quality at A_{260}/A_{280} ratio, which ranged from 1.8 to 1.9, and the quantity was directly displayed in nanogram per microlitres. The DNA samples were diluted to 50 ng/µl concentration as working stock and stored at –20°C. To measure the integrity of DNA, 0.8% agarose gel electrophoresis was used. Primers required for SSR analysis were synthesized from Eurofins, Bangalore. All primers were diluted by adding nuclease-free water equal to their concentration so that the final concentration was one nano mol/µl = 1000 p mol/µl in stock solution. By taking 5 µl each of forward and reverse primers from stock solution, and 90 µl of nuclease-free water, working solution of 10 p mol/µl was made. This working solution was further used for the PCR amplification in the SSR marker analysis. Parameters of DNA amplification (viz. specificity, efficiency and fidelity) are strongly influenced by the different components of the reaction and by thermal cycler set up[25]. Keeping this fact in view, careful optimization of these parameters will ultimately result in reproducible and efficient amplification. PCR conditions for pearl millet SSR primer pairs developed by previous researchers[26–31] were standardized with varying amounts and concentrations of chemicals for the PCR master mix, at different annealing temperatures. Additionally, for survey of SSR polymorphism between parents, further genotyping of PCRs was performed in a reaction volume of 15 µl. The PCR amplification cycle consisted of initial denaturation at 94°C for 5 min followed by a total of 39 cycles consisting of denaturation, primer annealing and extension at 94°C, 45–55°C and 72°C respectively, for 1 min each; and the final extension at 72°C for 7 min. PCR was carried out in a volume of 15 µl containing 50 ng/µl of template DNA, 1 µM each of forward and reverse primers, 2.5 mM deoxynucleotide triphosphates, 3 U/µl *Taq* polymerase and 1× of buffer with $MgCl_2$ in applied biosystem thermocycler. The amplified products were separated using 3% agarose gel stained with ethidium bromide. Scoring of the bands on the agarose gel was photographed using gel documentation system (Alpha Innotech Corporation, USA). The distance ran by amplified fragments from the well was translated to molecular size with reference to DNA ladder using FluorChem FC2 software.

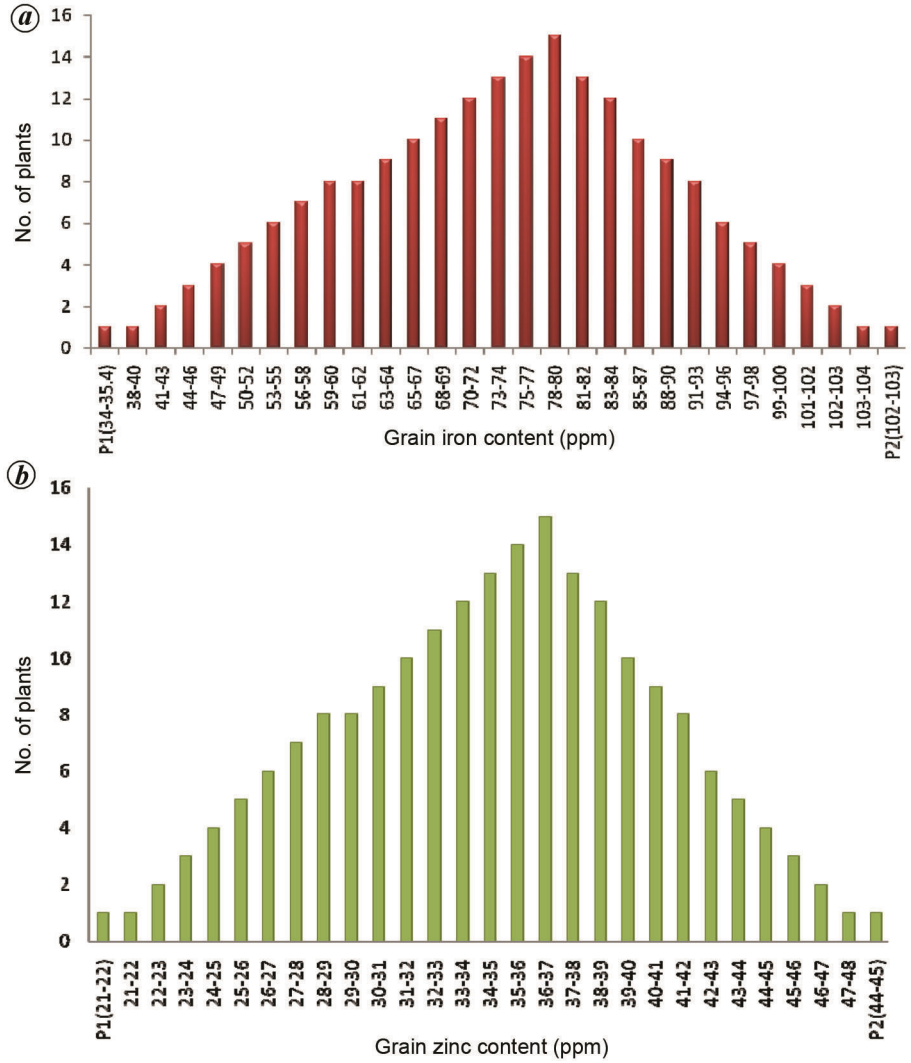

Figure 1. Frequency distribution of grain (***a***) iron content and (***b***) grain zinc content among the mapping population derived from the cross J 2340 and 30291.

Bulked segregant analysis

Parental polymorphism was surveyed between two contrasting parents using a total of 275 primers. The sequence information of 275 SSR primers used for polymorphism survey between the parents is given in Supplementary Table 1. The BSA was performed using the polymorphic markers identified from the parental polymorphism survey[22]. For BSA, equal quantities of DNA from 10 high Fe and Zn content lines, and 10 low Fe and Zn content lines of F_2 population was bulked individually to compose high and low bulks respectively[22]. The genotypes used for bulking of DNA in F_2 were similar for both the micronutrients content.

Results

Ninety nine out of 275 SSR primers (36%) showed polymorphism between phenotypically contrasting parents. The BSA was carried out to identify SSR markers linked to the gene/s with large effect on grain Fe and Zn content.

In the present study, frequency distribution of grain Fe and Zn content exhibited more or less normal distribution in a population of 201 individuals of $F_{2:3}$ population (Figure 1). The grain Fe and Zn content among the individuals of $F_{2:3}$ population, ranged from 38 to 103 ppm, and 22 to 47 ppm respectively. Progeny test results from $F_{2:3}$ families were used to determine the phenotype of F_2 plants. Two contrasting bulks were constituted from F_2 plants that formed two end tails of normal distribution graph.

In BSA, two SSRs, i.e. *Xipes0027* (on linkage groups 2 and 6) and *Xpsmp2263* (on linkage group 7) were found to be linked putatively with high Fe and Zn content. The primer *Xipes0027* showed a specific band of 214 bp in the high grain Fe and Zn content parent and the corresponding bulk, which was absent in contrasting parent and bulk (Figure 2). Similarly, the other tagged SSR, i.e.

Table 3. Information on linked SSR markers

Primer name	Repeat motif		Sequence 5′–3′	T_m (°C)	Guanine–cytosine (%)	Amplified product size (bp)
Xipes0027	$(GTT)_8$	F	TGCTTGGGACAAAAGGCT	53.7	50.0	214
		R	TAACTCAAGTGAGCGCAAGG	57.3	50.0	
Xpsmp2263	$(AG)_{33}$	F	AAAGTGAATACGATACAGGAGCTGAG	61.6	42.3	238
		R	CATTTCAGCCGTTAAGTGAGACAA	59.3	41.7	

T_m indicates melting temperature.

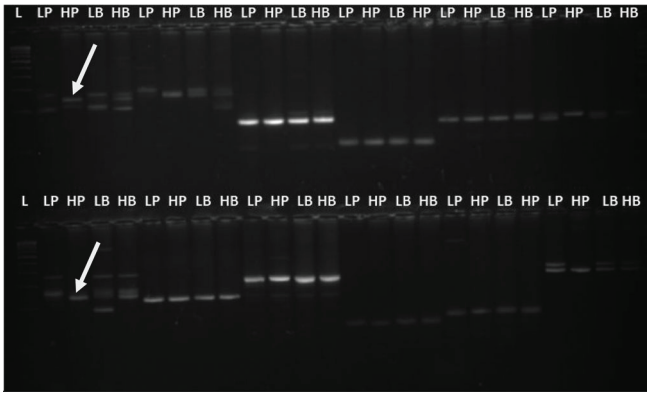

Figure 2. Amplification profiles of SSR primers for bulked segregant analysis: Upper and lower lane arrows indicate *Xipes0027* and *Xpsmp2263* SSR primers respectively, showing polymorphism between the parents and bulks. LP, Low grain iron and zinc content parent (J 2340); HP, High grain iron and zinc content parent (30291); LB, low grain iron and zinc content bulk; HB, high grain iron and zinc content bulk; L, 100 bp ladder.

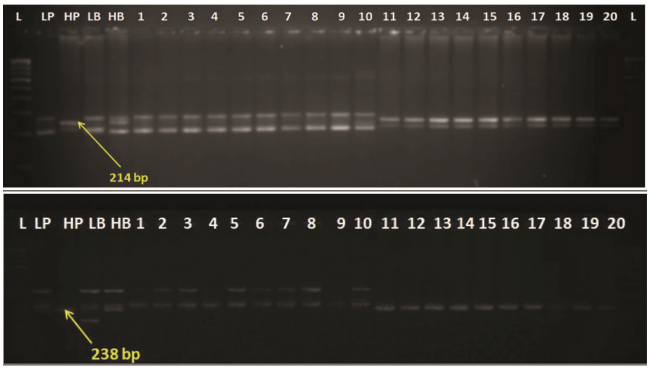

Figure 3. Co-segregation analysis of parents, bulks and F_2 mapping population. Upper and lower lane indicate co-segregation analysis of marker *Xipes0027* and *Xpsmp2263* respectively, with parents, bulks and progenies from F_2 mapping population. LP, Low grain iron and zinc content parent (J 2340); HP, High grain iron and zinc content parent (30291); LB, low grain iron and zinc content bulk; HB, high grain iron and zinc content bulk; 1–10, low grain iron and zinc content F_2 lines; 11–20, high grain iron and zinc content F_2 lines; L, 100 bp ladder.

Xpsmp2263 showed a specific band of 238 bp in the high grain Fe and Zn content parent and bulk, whereas the corresponding band was absent in the contrasting low parent and bulk (Figure 2).

The SSRs found to be polymorphic between parents and bulks were used to genotype individual F_2 plants (used for bulking) along with parents to further validate the results of BSA. Marker *Xipes0027* amplified band of 214 bp in high grain Fe and Zn content F_2 individuals was the same as in bulk and parent, whereas it was absent in low grain Fe and Zn content F_2 individuals, bulk and corresponding parent (Figure 3). Similarly, the primer *Xpsmp2263* amplified band of 238 bp in 10 high grain Fe and Zn content F_2 individuals, bulk and corresponding parent, was absent in the low grain Fe and Zn content parent, bulk and F_2 individuals (Figure 3). The results indicated that the linked markers *Xipes0027* and *Xpsmp2263* co-segregated with the phenotypic trait, i.e. high grain Fe and Zn content. The information of linked SSR markers is presented in Table 3.

Discussion

The grain Fe and Zn content in pearl millet is controlled by many genes with additive effects[32]. In the present study, frequency distribution of grain Fe and Zn content exhibited more or less normal distribution. The distribution of grain Fe and Zn content of different ranges could be used for further genetic improvement in pearl millet. The phenotypic normal distribution as well as presence of transgressive segregants for grain Fe and Zn content in recombinant infrared lines (RILs) population was also reported in pearl millet[16].

Bulk segregant analysis was originally designed to tag traits regulated by genes that had a significant effect and exhibited minimal genotype (G) × environment (E) interaction[19]. Recently, it has been modified to cover up the minor genes showing small effects which increased the power and efficiency of BSA in crop improvement programme.

In the present study, two SSR markers, viz. *Xipes0027* and *Xpsmp2263* were found to be putatively linked and reported to co-segregate with high grain Fe and Zn content. Here, the reported marker *Xipes0027* was present on linkage groups 2 and 6 of pearl millet, whereas *Xpsmp2263* was located on linkage group 7 according to the published SSR consensus maps. Both the markers used to tag high Fe and Zn content were newly identified and either linked to QTLs that were already identified, or they belonged to new gene/QTLs. Kumar *et al.*[16] also mapped two large effect QTLs for grain Fe and Zn content on linkage group 7 of pearl millet. Thus, *Xpsmp2263* of our study may either be linked to this QTL or other QTLs for high grain Fe and Zn content on the same chromosome. Moreover, till now, no QTLs for high grain Fe and Zn have been reported to be present on chromosome 2 or 6. Hence, the identified QTLs from this study may be new and need further validation. If QTLs reported in the present study are new, they may be taken up in the pyramiding programme to considerably improve grain Fe and Zn content in pearl millet. Similar studies were previously conducted by several researchers. The SSR markers, viz. *Xpsmp2261*, *Xipes0180* and *Xipes0096* were reported in pearl millet to be associated with high grain Fe and Zn content through association mapping[33]. QTLs analysis using SSR markers for grain Fe and Zn content was also carried out in pearl millet[16,34]. In congruence to the present findings, BSA using SSR markers was also used to identify the marker linked to gene or QTL for complex traits for water stress tolerance in wheat[35] and drought tolerance in rice[35–38]. In the present study, BSA was extended to identify the traits governed by minor genes having small and additive effects which increased the power and efficiency of this molecular technique in crop improvement programme. The identified SSR markers, i.e. *Xipes0027* and *Xpsmp2263* may be useful to screen higher grain Fe and Zn content genotypes in pearl millet in future crop improvement programmes.

Conflict of interest: The authors declare that they have no conflict of interest.

1. Varshney, R. K. *et al.*, Pearl millet genome sequence provides a resource to improve agronomic traits in arid environments. *Nat. Biotechnol*., 2017; doi:10.1038/nbt.3943.
2. AICPMIP Annual Report, Jodhpur, Rajasthan, India, 2020, 1–12; http://www.aicpmip.res.in/pcr2020.pdf
3. Kumar, M., Patel, M. P. and Rani, K., Delineating genetic inheritance and nonallelic genic interactions for grain iron and zinc concentration, yield and its attributes by generation mean analysis in pearl millet (*Pennisetum glaucum* (L.) R. Br.). *Genet. Resour. Crop Evol.,* 2021; http://doi.org/10.1007/s10722-021-01208-2
4. Vadez, V., Hash, T., Bidinger, F. R. and Kholova, J., Phenotyping pearl millet for adaptation to drought. *Front. Physiol*., 2012, **3**(386), 158–169.
5. Gupta, S. K. *et al.*, Seed set variability under high temperatures during flowering period in pearl millet (*Pennisetum glaucum* L. (R.) Br.). *Field Crops Res*., 2015, **171**, 41–53.
6. Deshmukh, D. S., Pawar, B. R., Yeware, P. P. and Landge, V. U., Consumer's preference for pearl millet products. *Agric. Update*, 2010, **5**(1–2), 122–124.
7. Kumar, M., Rani, K., Ajay, B. C., Patel, M. S., Mungra, K. D. and Patel, M. P., Multivariate diversity analysis for grain micronutrients concentration, yield and agromorphological traits in pearl millet (*Pennisetum glaucum* (L.) R. Br.). *Int. J. Curr. Microbiol. Appl. Sci*., 2020, **9**(3), 2209–2226; https://doi.org/10.20546/ijcmas.2020.903.253.
8. Kumar, M., Rani, K., Ajay, B. C., Patel, M. S., Mungra, K. D. and Patel, M. P., Study of genetic variability, heritability and path analysis for grain micronutrients concentration, yield and component traits in pearl millet (*Pennisetum glaucum* (L.) R. Br.). *J. Pharmacogn. Phytochem.,* 2020, **9**(2), 1402–1409.
9. Tako, E., Reed, S. M., Budiman, J., Hart, J. J. and Glahn, R. P., Higher iron pearl millet (*Pennisetum glaucum* (L.) R. Br.) provides more absorbable iron that is limited by increased polyphenolic content. *J. Nutr*., 2015, **14**, 11; doi:10.1186/1475-2891-14-11.
10. Deshpande, J. D., Joshi, M. M. and Giri, P. A., Zinc: the trace element of major importance in human nutrition and health. *Int. J. Med. Sci. Publ. Health*, 2013, **2**(1), 1–6; doi:10.5455/ijmsph.2013.2.1-6.
11. Stein, A. J., Global impacts of human malnutrition. *Plant Soil*, 2010, **335**, 133–154.
12. Bouis, H. E., Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? *Proc. Nutr. Soc*., 2003, **62**, 403–411.
13. Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M. and Shinozaki, K., Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biol*., 2011, **11**, 163.
14. El-Soda, M., Malosetti, M., Zwaan, B. J., Koornneef, M. and Aarts, M. G., Genotype × environment interaction QTL mapping in plants: lessons from *Arabidopsis*. *Trends Plant Sci*., 2014, **19**, 390–398.
15. Grishkevich, V. and Yanai, I., The genomic determinants of genotype environment interactions in gene expression. *Trends Genet*., 2013, **29**, 479–487.
16. Kumar, S. *et al.*, Mapping grain iron and zinc content quantitative trait loci in an iniadi-derived immortal population of pearl millet. *Genes*, 2018, **9**, 248; doi:10.3390/genes9050248.
17. Holland, J., Genetic architecture of complex traits in plants. *Curr. Opin. Plant Biol*., 2007, **10**, 156–161.

18. Xu, Y., Molecular dissection of complex traits: practice. In *Molecular Plant Breeding*, CABI, Wallingford, UK, 2010, pp. 249–285.
19. Zou, C., Wang, P. and Yunbi, Xu, Bulked sample analysis in genetics, genomics and crop improvement. *Plant Biotechnol. J*., 2016, **14**, 1941–1955; doi:10.1111/pbi.12559.
20. Sun, Y., Wang, J., Crouch, J. H. and Xu, Y., Efficiency of selective genotyping for genetic analysis of complex traits and potential applications in crop improvement. *Mol. Breed*., 2010, **26**, 493–511.
21. Xu, Y. and Crouch, J. H., Marker-assisted selection in plant breeding: from publications to practice. *Crop Sci*., 2008, **48**, 391–407.
22. Michelmore, R. W., Paran, I. and Kesseli, R. V., Identification of markers linked to disease resistance genes by bulked-sergeants analysis; a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 9828–9832.
23. Singh, D., Chhonkar, P. K. and Dwivedi, B. S., Manual on Soil, Plant and Water Analysis, Westville Publishing House, New Delhi, 2005.
24. Murray, M. G. and Thompson, W. F., Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res*., 1980, **8**, 4321–4326; doi:10.1093/nar/8.19.4321.
25. Caetano-Anolles, G., Nucleic scanning by amplification with mini-hairpin and microsatellite oligonucleotide primers. In *DNA Markers: Protocols, Applications and Overviews* (eds Caetano-Anolles, G. and Gresshoff, P. M.), Wiley-Liss, Inc., New York, 1997, pp. 91–114.
26. Allouis, S., Qi, X., Lindup, S., Gale, M. D. and Devos, K. M., Constructions of BAC library of pearl millet, *Pennisetum glaucum*. *Theor. Appl. Genet*., 2001, **102**, 1200–1205.
27. Budak, H. F., Pedraza, P. B., Cregan, P. S., Baenziger and Dweikat, I., Development and utilization of SSRs to estimate the degree of genetic relationships in a collection of pearl millet germplasm. *Crop Sci*., 2003, **43**, 2284–2290.
28. Qi, X., Lindup, S., Pittaway, T. S., Allouis, S., Gale, M. D. and Devos, K. M., Development of simple sequence repeat markers from bacterial artificial chromosomes without subcloning. *Biotechniques*, 2001, **31**, 355–362.
29. Qi, X. *et al.*, An integrated genetic map and new set of simple sequence repeat markers for pearl millet. *Pennisetum glaucum*. *Theor. Appl. Genet*., 2004, **109**, 1485–1493.
30. Rajaram, V. *et al.*, Pearl millet (*Pennisetum glaucum* (L.) R. Br.) consensus linkage map constructed using four RIL mapping populations and newly developed EST–SSRs. *BMC Genomics*, 2013, **14**, 159.
31. Senthilvel, S., Jayashree, B., Mahalakshmi, V., Kumar, P., Nakka, S., Nepolean, T. and Hash, C. T., Development and mapping of simple sequence repeat markers for pearl millet from data mining of expressed sequence tags. *BMC Plant Biol*., 2008, **8**, 119.
32. Kanatti, A., Rai, K. N., Radhika, K., Govindaraj, M., Sahrawat, K. L. and Rao, A. S., Grain iron and zinc density in pearl millet: combining ability, heterosis and association with grain yield and grain size. *SpringerPlus*, 2014, **3**, 763–774.
33. Anuradha, N. *et al.*, Deciphering genomic regions for high grain iron and zinc content using association mapping in pearl millet. *Front. Plant Sci*., 2017, **7**; https://doi.org/10.3389/fpls.2017.00412.
34. Kumar, S. *et al.*, Mapping quantitative trait loci controlling high Fe and Zn density in self and open pollinated grains of pearl millet (*Pennisetum glaucum* (L.) R. Br.). *Front. Plant Sci*., 2016, **7**, 1636; doi:10.3389/fpls.2016.01636.
35. Altinkut, A. and Gozukirmizi, N., Search for microsatellite markers associated with water–stress tolerance in wheat through bulked segregant analysis. *Mol. Biotechnol*., 2003, **23**, 97–106.
36. Bernier, J., Kumar, A., Ramaiah, V., Spaner, D. and Atlin, G., A large effect QTL for grain yield under reproductive stage drought stress in upland rice. *Crop Sci*., 2007, **47**, 507–516.
37. Kanagaraj, P., Prince, K. S. J., Sheeba, J. A., Biji, K., Paul, S. B., Senthil, A. and Babu, R. C., Microsatellite markers linked to drought resistance in rice (*Oryza sativa* L.). *Curr. Sci*., 2010, **98**, 836.
38. Venuprasad, R., Dalid, C. O., Valle, M. D., Zhao, D., Espiritu, M., Cruz, M. T. S. and Amante, M., Identification and characterization of large-effect quantitative trait loci for grain yield under lowland drought stress in rice using bulk segregant analysis. *Theor. Appl. Genet*., 2009, **120**, 177–190.

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