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Molecular mapping of quantitative trait loci for zinc, iron and protein content in the grains of hexaploid wheat

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Abstract Understanding the genetic basis of micronutrient concentration in wheat grain may provide useful information to breed for biofortified varieties through marker assisted selection (MAS). One hundred and thirty eight doubled haploid progeny of a cross between the wheat cultivars 'Berkut' and 'Krichauff' were evaluated for 2 years at two locations on the eastern Gangetic plains of India under timely (November) sown conditions. Grains were evaluated for Zn and Fe concentrations by energy-dispersive X-ray fluorescence. Using composite interval mapping, three QTLs were identified; two for Zn (1B and 2B) with a QTL (2B) co-located for Fe and the third (1A), for protein. The QTL located on chromosome 1B (flanked by *wmc036–cfa2129*) and 2B (flanked by *gwm120–wpt2430*) for Zn explained up to 23.1 and 35.9 % of mean phenotypic variation respectively, whereas up to 22.2 % was explained by the Fe QTL co-located with the Zn QTL on chromosome 2B. A QTL for grain protein was detected on chromosome 1A and flanked by the markers, *wpt9592* and *GBM1153* which explained up to 17.7 % of the total phenotypic variation. With their detection over consecutive seasons, the detected QTLs appeared robust and useful for MAS.

Keywords *Triticum aestivum* · Hexaploid wheat · QTL · X-ray fluorescence · Micronutrients · Protein · Iron and zinc content

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Introduction

Micronutrient malnutrition, particularly the deficiency in Zn and Fe affects over three billion people worldwide (Welch and Graham 2004; Bouis 2007), resulting in overall poor health, anemia, increased morbidity and mortality rates, and lower worker productivity (Cakmak 2002). This problem is significantly higher in South Asia and Africa compared to other parts of the world. In South Asia, wheat is a major staple crop for 1.6 billion people, contributing around 20.0 % of the daily calorie intake, 20.4 % of

total protein and 18.8 % of total energy supply. Considering the importance of hexaploid wheat (*Triticum aestivum*) as a staple crop, even a small increase in its nutritional value can help to decrease deficiencies of key micronutrients, especially Zn and Fe (Graham et al. 2007).

Zn and Fe concentrations in wheat grains have been reported to be unstable and vary across locations and years (Joshi et al. 2010). Their concentration in the wheat grain have been reported to depend largely on environmental conditions, particularly soil composition (Velu et al. 2012). Another reason for high degree of variability is their quantitative inheritance as reported by Shi et al. (2008) and Srinivasa et al. (2014). Since grain Zn and Fe concentrations are controlled by multiple genes, their accumulation in seeds becomes a complex multigenic phenomenon. Therefore increasing micronutrient concentration using conventional breeding becomes a difficult task (Velu et al. 2012). Some QTLs for grain Zn and Fe concentrations were reported by different workers during last few years (Distelfeld et al. 2007; Ozkan et al. 2007; Shi et al. 2008; Peleg et al. 2009; Tiwari et al. 2009; Srinivasa et al. 2014).

Grain protein content (GPC) in wheat (*Triticum aestivum* L.) is another important trait for human nutrition. The GPC has been the most successful and extensively studied marker, however this trait was reported to have a negative correlation with grain yield (Simmonds 2006). Therefore QTLs with less negative impact on yield are required.

Advancements in QTL mapping facilitate an understanding of the genetic basis controlling micronutrient concentrations in wheat grains. Furthermore, the identification and tagging of major QTLs for the traits in relation to micronutrients with large effects will be helpful in the selection of the QTLs in early generations with marker assisted selection (MAS) technique, and will greatly accelerate wheat cultivar development (Ortiz-Monasterio et al. 2007).

The present study was carried out to map genes for grain zinc, iron and protein concentrations using a double haploid population from a wheat cross "Berkut" × "Krichauff". Evaluation of the population was done under environmental conditions of the eastern Gangetic plains (EGP) of India.

Materials and methods

Plant material

The doubled-haploid (DH) population comprised of 138 segregants from the cross cv. 'Berkut' × cv. 'Krichauff'. The cultivar 'Berkut' was derived from the cross IRENE/BAV92//PASTOR while 'Krichauff' from WARIQUAM//KLOKA/PITIC-62/3/WARIMEK/HALBERD/4/3-AG-3/AROONA. 'Berkut' was developed at CIMMYT (2002) and is low in Zn, Fe and protein content, while cv. 'Krichauff' is an Australian cultivar with significantly higher concentrations of these nutrients. This population has already been used for other traits in earlier studies (McDonald et al. 2008; Huynh et al. 2008; Genc et al. 2010; Nguyen et al. 2011; Tiwari et al. 2013).

Crop management

The field experiments were raised at the Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (N25°15.293′, E082°59.014′) and Jamalpur, Mirzapur (N25°08.541′, E083°04.850′) over two consecutive crop seasons (2011–2012 and 2012–2013). The population was planted using a randomized complete block design with six row plots of length 3 m and an inter-row spacing of 25 cm. Each of the two replications for each DH line were comprised of four blocks, each of which included 35 entries plus a local check variety (HUW 234) planted after every fifth entry. The mean performance of each contiguous pair of check plots was used as a covariate for the entries sown between them (Tiwari et al. 2013).

The crop was irrigated thrice, and the fertilizer application followed local commercial practice (120 kg N, 60 kg P_2O_5, 40 kg K_2O per ha); the P_2O_5 and K_2O were applied in one shot during sowing, but the N application was split such that one third was given at sowing, and a further one third after each of the first and the second irrigations. The crop was protected from infection by spot blotch and leaf rust by spraying with 625 g/ha Tilt (propiconazole) at growth stages GS54 and GS69 (Zadoks et al. 1974), and was maintained weed-free by applying 1 kg/ha pendimethalin 3 days after sowing, followed by 25 g/ha sulfosulfuron 25 days after sowing. Soil Zn and Fe

concentrations were mapped in each of the locations by following standard procedure.

Data collection

Zn, Fe and protein concentration were analysed using the grains of hand threshed, 100 spikes in gunny bags from each plot. Before analysis, grains were cleaned to avoid any dust or contamination that could influence the analysis. The Zn and Fe (ppm) analysis was done using X-ray Fluorescence (EDXRF spectrometer X-Supreme 8000, (Paltridge et al. 2012). The FOSS (Infra-tech 1241) grain analyser was used to estimate the protein content of the grains.

Linkage map construction and QTL detection

A linkage map was constructed using Map Manager vQTXb20 (Manly et al. 2001) by applying the Kosambi mapping function and setting a threshold P value of 0.01. The genotypic data (233 SSRs, 311 DArT and 1 *Vrn* gene linked marker) was initially arranged into groups using the "Make Linkage Groups" command, and extra markers were then integrated using the "Links report" command; thereafter, the "ripple" function was applied to minimize the number of double recombinants and the chromosome length. Marker order was verified using RECORD, with ripples = 0, EQV threshold = 0 (Van Os et al. 2005). Goodness-of-fit to the expected 1:1 segregation between the cv. 'Berkut' and the cv. 'Krichauff' allele was tested using a χ^2 test ($P < 0.05$). QTL Cartographer v2.5 (Wang et al. 2007) was employed to identify QTL. The parameters used for CIM were based on model 6, with a forward and a backward stepwise regression (threshold P value <0.05) to select cofactors, a window size of 10 cM, and a 2 cM walking speed along the chromosome. The LOD score for declaring a QTL was based on 1000 permutations and hence varied for different traits. The QTL were named in the manner recommended by http://wheat.pw.usda.gov/ggpages/wgc/98/: each consisted of the letter Q, followed by an abbreviated trait name, the institution designation (bhu) and the identity of the chromosome involved.

Statistical analysis

Analysis of variance and the calculation of phenotypic correlation coefficients were performed using GenStat-12.1/2009 (www.vsni.co.uk/software/genstat) software. Heritability's (h^2) were estimated from the expression $\left[\sigma_g^2 \Big/ \left(\sigma_g^2 + 1/t\ \sigma_{gt}^2 + 1/rt\ \sigma^2\right)\right]$, following (Nyquist and Baker 1991); here, σ_g^2 represented the genotypic variance, σ_{gt}^2 the genotype × trial variance, σ^2 the residual variance, r (3) the number of replications and t (6) the number of trials.

Results

Phenotypic analysis

The cultivar 'Krichauff' had significantly higher Zn, Fe and protein compared to the other parent 'Berkut' (Fig. 1a–c). Variance effects of genotype and environment on grain Zn, Fe and protein concentration in DH lines grown under four environments were found significant. The range for Zn was 28.7–40.8 ppm, for Fe 33.6–46.3 ppm while for protein concentration it was 12.5–16.5 %.Broad sense heritability of grain Zn, Fe and protein was moderate to high as 53, 72 and 78 % respectively (Table 1). These three traits showed continuous distribution like quantitatively inherited traits and were positively correlated with each another (Table 3). Soil composition for mean Zn in BHU location over 2 years was 1.20 ppm while at and Jamalpur, it was 0.98 ppm. Likewise, mean Fe of BHU soil in 2 years was 90.06 ppm while 119.20 ppm at Jamalpur

QTL analysis

Of the 1150 SSR assays applied in the parental screen of cvs. 'Berkut' and 'Krichauff', 233 were informative. Of these, 17 could not be assigned to any of the linkage groups and 11 failed to segregate consistently with the expected 1:1 ratio. The QTL analysis is presented in Figs. 2a–d and 3. Using CIM, three stable QTLs were detected, mapped to chromosomes 1B, 2B and 1A (Table 2).Two major QTLs mapped for Zinc were *QZn.bhu-1B* flanked by *wmc036c* and *cfa2129,* and *QZn.bhu-2B* flanked by *gwm120* and *wpt2430*. The year-by-year LOD scores for QTL *QZn.bhu-1B* varied from 2.9 to 5.9 while phenotypic variance (%PVEs) ranged 11.8–27.4 %. Across the four environments, the LOD score was 5.0 and the %PVE 23.1.

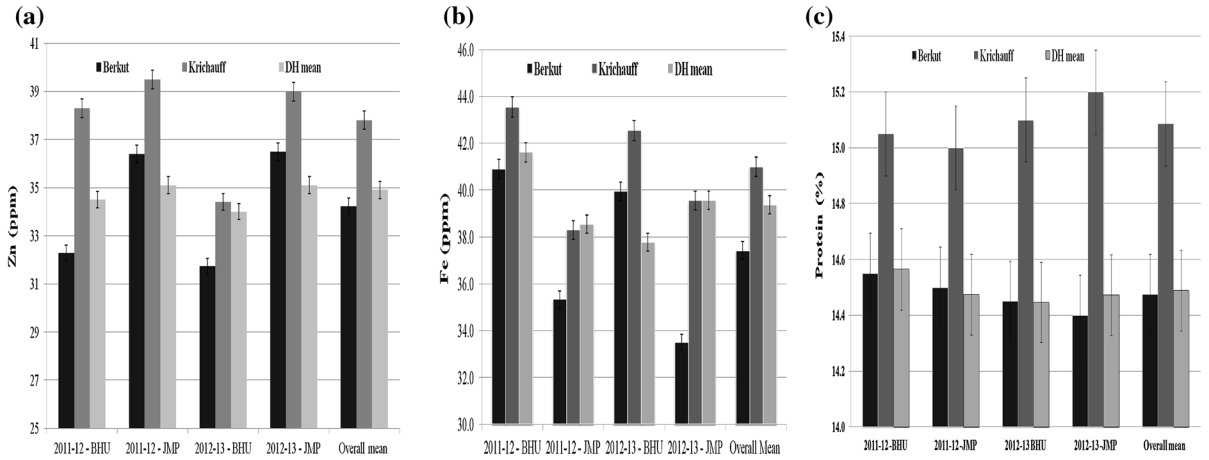

Fig. 1 **a–c** Distribution of grain Zn, Fe and protein concentrations of a Berkut × Krichauff DH wheat population grown in the years 2011–2013

Table 1 Analysis of variance (ANOVA) and heritability (h2) of grain zinc, iron and protein in a Berkut × Krichauff double haploid population of wheat tested for 3 years in EGP of India

Source of variation	df	Zinc	Iron	Protein
Varieties	139	45.7**	21.7**	2.8**
Rep within Env	4	9.1**	49.9**	1.5*
Environments	3	12.4**	334.1**	223.4**
Var.*Env	417	0.6**	3.7**	0.6**
Pooled error	556	4.3	3.1	0.4
LSD		3.6	1.8	0.5
Heritability %		53	72	78
Range		28.7–40.8	33.6–46.3	12.5–16.5

* Significant at P = 0.05, ** significant at P = 0.01

The QTL *QZn.bhu-2B* having a LOD score of 3.0 over environments is detected on chromosome 2B which is flanked by the marker *gwm120* and *wpt2430* with an interval of 6.5 cM. This QTL explained 23.2–30.3 % of phenotypic variation across four environments with a mean of 35.9 % (Table 2; Fig. 2a–b). The allele for improved Zn was inherited from cv. 'Krichauff'.

A major QTL *QFe.bhu-2B* for Fe concentration was mapped between *gwm120* and *wpt2430* with an interval of 6.5 cM which explained 14.7–37.7 % of the phenotypic variation. The superior allele was inherited from cv. 'Krichauff'. The QTL detected on chromosome 2B between the flanking markers *gwm120* and *wpt2430* found to control variation for both Zn and Fe concentrations with a mean LOD score of 8.5 and explained up to 22.2 % of phenotypic variation (Table 2; Fig. 2c).

The third QTL *QGPC.bhu-1A* was mapped between *wpt9592* and *GBM1153* (3.5 cM), the LOD score ranged from 2.8 to 2.9 and %PVE 16.2 to 17.7 respectively across the years. The %PVE over years was 17.7 with LOD of 3. The source of the favorable allele for *QGPC.bhu-1A* was cv. 'Krichauff' (Table 2; Fig. 2d). QTL × Environment interaction was non-significant for all the traits (Table 2).

Discussion

In the present experiment, grain Zn, Fe and protein concentrations showed a continuous distribution in the DH population, hence the traits appeared quantitatively inherited. The recovery of higher concentration of micronutrients and protein in the progenies was due to transgressive segregation suggesting that both parents carried a few different genes with allele contributing to increased Zn and Fe concentration (Ozkan et al. 2007; Xu et al. 2012). Variation in recombinant inbred lines for Zn, Fe and protein were earlier reported (Tiwari et al. 2009; Cakmak et al. 2004; Srinivasa et al. 2014).

Cultivated hexaploid wheat germplasm has a narrow range for grain Fe and Zn concentrations (Tiwari et al. 2009). However, in the DH lines investigated, a substantial range of variation was observed; for Zn 28.7–40.84 ppm for Fe 33.6–46.3 ppm and for protein 12.5–16.5 %. Morgounov et al. (2007) also reported a substantial variation for grain Zn and Fe concentrations in a set

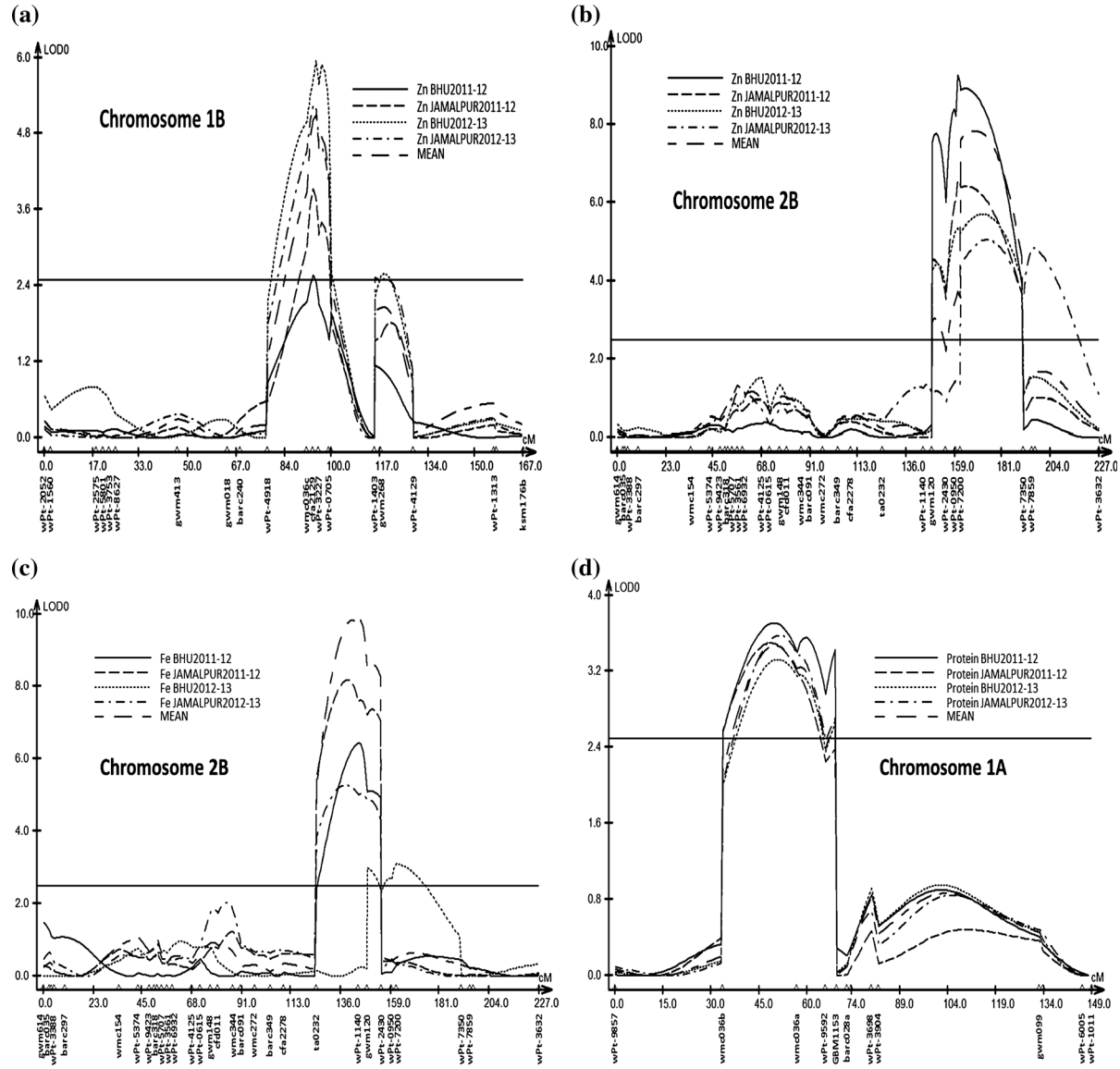

Fig. 2 **a–d** Chromosomal location and logarithm of odds (LOD) scores of grain Zn, Fe and protein concentration in a Berkut × Krichauff population tested during 2011–2013

of spring and winter wheat cultivars. Broad sense heritability of grain Zn, Fe and protein was observed as moderate to high. Velu et al. (2012) and Srinivasa et al. (2014) also reported medium to high broad sense heritability for grain Zn and Fe across nine environments of south Asia and Mexico. This assumes significance for breeding biofortified wheat varieties.

The QTL *QZn.bhu-1B* for Zn concentration, mapped on chromosome 1B, had the flanking markers *wmc036c–cfa2129*. Hao et al. (2014) also got QTL on the same chromosome for Zn content in the RILs from the cross PBW343/Kenya Swara. Shi et al. (2008) also detected 4 QTLs for Zn concentration and 3 for Zn content where a QTL on chromosome 7A explained the highest phenotypic variation. Xu et al. (2012) found one QTL for Zn on 3D. The *QTL* (*QZn.bhu-2B*) mapped on 2B in this study was also detected by Velu et al. (2013) with the same DArT marker *wpt2430* while studying landraces and wild relatives of wheat. It is of interest that the results were consistent across

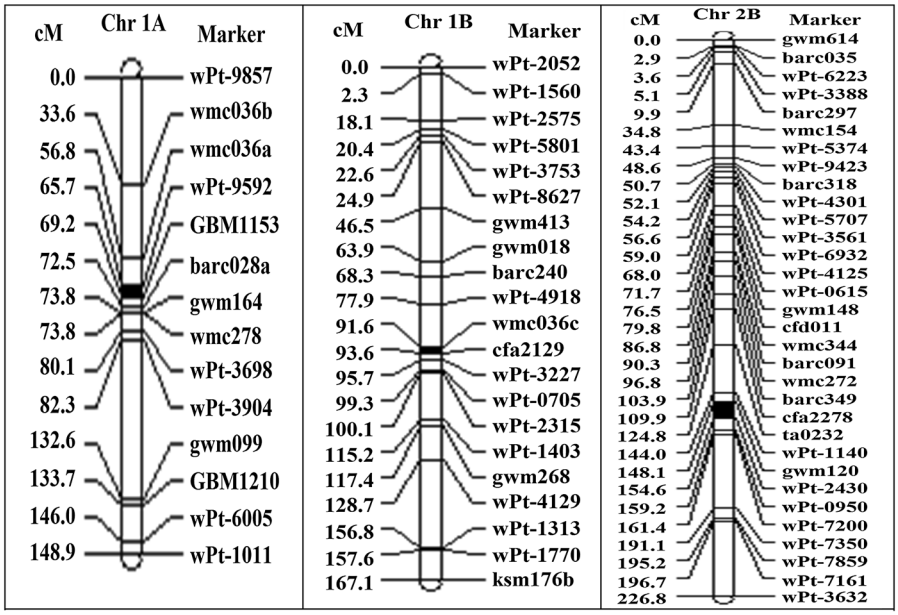

Fig. 3 Location of QTLs for grain Zn, Fe and protein concentration. QTLs are indicated by *dark black filled* position of each chromosome

different populations and the QTL co-localized with Fe concentration as well. Srinivasa et al. (2014) also detected a Zn QTL on chromosome 2B with 16.5 % PVE in RILs of *T. spelta* × *T. aestivum* cross. In addition, they also found other Zn QTL on 2A, 3D, 6A and 6B.QTL for Zn concentration on chromosome 2A co-localized with a QTL for Fe concentration Srinivasa et al. (2014). Tiwari et al. (2009) also detected colocalization between markers *Xgwm473–Xbarc29* on chromosome 7A for Zn and Fe concentration in wheat. The co-localization of grain Zn and Fe QTL has also been observed in tetraploid wheat (Peleg et al. 2009). If Zn and Fe concentrations are co-localized it provides a common genetic basis for grain Zn and Fe concentration in wheat, suggesting the traits can be combined and improved simultaneously (Welch and Graham 2004; Tiwari et al. 2009; Genc et al. 2009; Xu et al. 2012).

The QTL obtained for Fe concentration in chromosome 2B was also obtained by Yasmin et al. (2013) using the same DH population tested in South Australia. However, the DArT markers (*wPt-4209* and *wPt-5390*) were different suggesting that different loci for Fe were identified which might be allelic. Tiwari et al. (2009) identified two QTLs for grain Fe on chromosomes 2A and 7A and a robust QTL (*QGPC.bhu-1A*) for GPC which explained 17.7 % PVE. Earlier reports (Perretant et al. 2000; Blanco et al. 2002) also suggested several QTLs for GPC. They (Perretant et al. 2000; Blanco et al. 2002) also found variable responses of QTLs across environments and concluded that data should be collected over a range of locations to identify putative QTLs. Correlation coefficients are low (cf. Table 3) that resulted in coefficient of determination, denoted R2 or r2, lower than 25 %; therefore, a direct selection for the individual traits seems not very successful.

Biofortification of wheat can be achieved through organized plant breeding without affecting the yield or quality (Velu et al. 2012). It is also a more sustainable and cost-effective solution (White and Broadley 2005). Significant knowledge has been gained on the molecular mechanisms affecting the accumulation of Fe (Bauer et al. 2004; Cakmak 2002) and Zn (Hacisalihoglu and Kochian 2003) in plants. The consistent data of Zn, Fe and protein concentration found across four environments in the present study is like that obtained by Velu et al. (2012) and Tiwari et al. (2009). Multi environment experiments in wheat have led to the recognition of a number of QTLs underlying grain micronutrient concentration which are effective across a range of environments (Peleg et al. 2009; Tiwari et al. 2009; Velu et al. 2012). The non-significant QTL × Environment interaction for all the traits showed stability of the QTLs and such loci are prime targets for MAS. The difficulty of using conventional breeding to improve grain mineral contents means that MAS would be an attractive

Table 2 Quantitative trait loci (QTL) for grain Zn, Fe and protein concentration of Berkut × Krichauff doubled-haploid population grown at EGP of India in 2011–2013

Traits/QTL	Marker Interval	Int Size (cM)	Chromosome	2011–2012						2012–2013						Mean			
				B.H.U., Varanasi			Jamalpur, Mirzapur			B.H.U., Varanasi			Jamalpur, Mirzapur						
				LOD	% PVE	% ADD	LOD	% PVE	% ADD	LOD	% PVE	% ADD	LOD	% PVE	% ADD	LOD	% PVE	% ADD	QTL*E
GZnC																			
QZn.bhu-1B	*wmc036c–cfa2129*	2	1B	2.9	11.8	0.4	3.9	27.4	0.4	5.9	11.9	0.3	5.2	24.1	0.4	5.0	23.1	0.4	NS
QZn.bhu-2B	*gwm120–wpt2430*	6.5	2B	7.7	24.0	0.5	4.5	30.3	0.4	4.3	26.3	0.4	3.0	23.2	0.4	3.0	35.9	0.4	NS
GFeC																			
QFe.bhu-2B	*gwm120–wpt2430*	6.5	2B	5.0	29.6	0.2	7.3	37.7	0.4	3.0	14.7	0.2	4.7	35.0	0.2	8.5	22.2	0.4	NS
GPC																			
QGPC.bhu-1A	*wpt9592–GBM 1153*	3.5	1A	2.9	17.7	0.3	2.8	16.4	0.1	2.8	16.2	0.3	2.8	17.1	0.3	3	17.7	0.3	NS

%PVE = percentage of phenotypic variance explained; * Significant at P = 0.05; Add: additive effect, where a positive value indicates that the cv. 'Krichauff' allele was favorable and negative value that cv. 'Berkut' allele was favorable; LOD = Likelihood of odds ratio

Table 3 Pearson correlation coefficient of measured grain Zn, Fe and protein concentration in the Berkut × Krichauff DH population of wheat

Trait	Zn	Fe	Protein
Zn	1	0.43(**)	0.35(**)
Fe		1	0.50(**)
Protein			1

proposition, provided that robust QTL can be identified as in the case of many other traits of wheat (Gupta et al. 2010). The consistency of some of the QTL identified here represents potential candidates for marker development and subsequent marker–assisted breeding.

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