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Assessment of genetic biodiversity and association of micronutrients and agronomic traits using microsatellites and staining methods which accelerates high-micronutrients variety selections within different wheat groups

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Evaluation of genetic biodiversity for micronutrients is crucial for breeding high-quality crops and addressing the negative impacts of mineral deficiencies. The objectives of this research were to assess genetic variation and the relationship between grain Fe and Zn levels and agronomic traits in a diverse collection of wheat varieties. Additionally, the study aimed to determine the correlation between microsatellite markers (SSR) and micronutrient quantities. A total of 42 genotypes (Iranian commercial cultivars, landraces, and Afghan and Swiss varieties) were evaluated over a two-year period. Fe and Zn levels were measured using two semi quantitative staining assays and atomic absorption spectrophotometry (AAS) facility. Semi-quantitative staining methods and AAS showed high correlations for micronutrient contents. Landraces exhibited higher Fe (63.79 mg/kg) and Zn (44.76 mg/kg) but lower grain yield compared with commercial cultivars. Heritability estimates ranged 53%-79.43%, suggesting that genetic variance played a higher contribution in the phenotypic variation of traits than environmental factors. Notably, Fe content displayed significant correlations with days to maturity. Canonical correlation analysis (CCA) revealed that Zn content was correlated with four agronomic traits. Evaluation of genetic diversity using SSR markers demonstrated high genetic variation among the genotypes tested. The analysis of polymorphism information content (PIC) indicated that SSR primers had an average PIC of 0.75, with the *Xgwm192* primer exhibiting higher PIC than others. Several SSR markers revealed association with micronutrient content that can be used in marker-assisted selection (MAS) programs aimed at selection of high micronutrient genotypes. In conclusion, the findings underscored the substantial genetic diversity present in micronutrient levels among global wheat genotypes, the potential of landraces for micronutrients biofortification of wheat cultivars through cross hybridization, the utility of staining methods for screening high/low micronutrient genotypes, and use of microsatellite markers for marker-assisted breeding aiming to micronutrient improvement in breeding programs.

Keywords Atomic absorption, Canonical correlations, Landrace, Staining method, Wheat

Wheat, the most important staple crop, covers 220 million hectares of agricultural lands worldwide, with grain production exceeding 750 million tons in 2019[1,2]. While wheat is commonly known as a source of starch and energy, it also provides significant amounts of essential components such as proteins, vitamins, minerals, and dietary fiber that are crucial for human health[2–5]. The quality of grain is also vital for human health in countries

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with a cereal-based diet[6–8]. It is estimated that 60–80% of people are affected by iron deficiency and more than 30% are zinc deficient, which can be addressed by consuming foods rich in micronutrients[7,9]. With the increasing population, urbanization, and globalization, the consumption of wheat products, especially in Asia, is steadily rising[10,11]. To meet the growing population and food demand, increased grain yield must be accompanied by high-quality grain, which is directly related to the presence of micronutrients in wheat[12]. Micronutrients such as zinc, iron, manganese, and copper play crucial roles in plant growth and development, as well as in animal and human health[13–16].

The challenge of screening a large number of samples in plant breeding programs and the high cost of quantifying micronutrients have impeded research on breeding for micronutrients in wheat. Various methods, such as staining, atomic absorption spectrophotometer (AAS), and inductively coupled plasma (ICP) spectrophotometer, have been utilized for quantifying micronutrients[17]. To analyze a significant number of genotypes for micronutrient content, replacing AAS and ICP methods with staining techniques like Pearl's Prussian Blue (PPB) and diphenyl-thio-carbazone (DTZ) could offer a more accessible and cost-effective alternative that saves time and energy[18]. The PPB staining method is commonly used for estimating Fe content, while DTZ is typically used for Zn content in grains[17,19,20].

Numerous strategies have been developed to increase micronutrient levels in plants in order to address global malnutrition issues. These strategies include seed priming, foliar application, soil treatment, biofortification, and breeding methods[21–38]. However, breeding methods that involve genetic diversity and selection of plants with higher micronutrient levels have proven to be a sustainable and cost-effective approach to achieving high micronutrient plants and a balanced diet[39,40]. Studies evaluating genetic diversity have revealed significant variability in micronutrient content, particularly zinc (Zn) and iron (Fe) in wheat[5,41–43]. The strong correlation between grain yield and Zn and Fe contents indicates that genetic-based biofortification can be achieved without sacrificing yield[44,45]. The use of DNA markers to screen plants with high Zn and Fe contents shows promise due to its independence from environmental factors and its ability to provide more reliable estimates of genetic distances[46]. For example, a study analyzed 11 high-yield wheat genotypes using SSR markers linked to micronutrients[47]. In another study conducted in Mexico, 62 advanced lines were examined for grain yield, Zn, and Fe content in Zn-enriched soils over two growing seasons, revealing high genetic variability in Zn and Fe contents[48]. Interestingly, a positive correlation was found between Zn and Fe in wheat commercial cultivars in the same study. Additionally, a comparative analysis of landraces and commercial wheat cultivars demonstrated higher Zn, Fe, and grain yield in landraces compared to commercial genotypes[49]. Furthermore, the results of a study by Heidari et al. [50] indicated a strong correlation between grain protein and Zn and Fe contents in wheat. Despite this, in recent decades, breeding programs have primarily focused on developing wheats with maximum grain yield, with limited attention given to the concentration of micronutrients in commercial wheat grains[51–53]. However, wild relatives and progenitors of cultivated wheat possess significant genetic variation for micronutrients, such as Zn and Fe, which could be leveraged in breeding programs to enhance the micronutrient content of wheat grains[41,54,55].

Despite the potential of landraces for improving traits in commercial varieties, little attention has been given to utilizing landrace gene pools in breeding for micronutrients in wheat. Potential of landraces has been almost assessed for disease resistance genes and agronomic traits and such gene pools have not been adequately explored for their micronutrient content[49]. Additionally, accessing rapid and cost-effective measures of micronutrients in large sample sizes has been a challenge in breeding programs. DNA markers improve the productivity and accuracy of classical plant breeding by means of marker-assisted selection (MAS). Marker-assisted selection is a newly emerging approach due to which various problems of conventional breeding avoid and enhance the selection criteria of phenotypes with the selection of genes, either indirectly or directly. DNA markers are not regulated through the environment and not affected by conditions in which the crop plants are grown. Therefore, the objectives of this study were to (1) evaluate genetic biodiversity of micronutrients through both micronutrients phenotypic data in the field and polymorphisms in SSR markers, (2) determine the relation of color image analysis using staining methods as rapid, simple and user friendly approach with traditional AAS which incurs time and cost for quantifying micronutrients, and (3) analyze the relationship of microsatellite (SSR) markers and micronutrients for use in marker-assisted selection (MAS) programs for the improvement of micronutrients in a diverse core collection of wheat genotypes.

Methods

Plant material and experimental design

The plant materials consisted of Iranian commercial cultivars and landraces, and Afghan and Swiss varieties (Table 1). A randomize complete block design (RCBD) with three replications was conducted during 2015-2016 and 2016-2017 growing seasons at the research field of School of Agriculture, Shiraz University, Iran. In each growing season, seeds were manually sown on November in 1-2 cm depth in 40 × 100 cm plots with seed spaces of 5 cm. All recommended agronomic practices were followed during growth season. Prior to sowing, 100 kg ha^{-1} potassium (K_2So_4) and 150 kg ha^{-1} triple superphosphate 250 kg ha^{-1} were incorporated with the soil. The nitrogen fertilizer top dressed in three stages including pre-sowing, tillering and flowering. Watering plants was performed through flood irrigation method according to soil moisture content. Weeding was done during growing cycle and no specific pesticides were used. Information of soil elements and weather data are presented in the Supplementary Table 1.

Agronomic traits

Several agronomic traits including plant height (cm), length of main spike, and spikelet number per spike were measured in 10 plants on each plot in the field. Days to maturity were recorded when grains in more than 50%

Code	Genotype	Type	Code	Genotype	Type	Code	Genotype	Type
1	Arina	Afghan Vr	15	KC4537	Iranian Landrace	29	S-B120	Swiss Vr
2	Bayat	Iranian Cv	16	KC4542	Iranian Landrace	30	S-B122	Swiss Vr
3	Butshak	Afghan Vr	17	KC4551	Iranian Landrace	31	S-B126	Swiss Vr
4	Camedo	Swiss Vr	18	KC4557	Iranian Landrace	32	S-B136	Swiss Vr
5	Danesh	Iranian Cv	19	KC4633	Iranian Landrace	33	S-B136	Swiss Vr
6	Dez	Iranian Cv	20	KC4848	Iranian Landrace	34	S-B15	Swiss Vr
7	Falat	Iranian Cv	21	KC4863	Iranian Landrace	35	S-B21	Swiss Vr
8	Forno	Swiss Vr	22	Kutschos	Iranian Cv	36	S-B29	Swiss Vr
9	Iran811	Iranian Landrace	23	Mahdavi	Iranian Cv	37	S-B74	Swiss Vr
10	Iran906	Iranian Landrace	24	Maroun	Iranian Cv	38	S-B79	Swiss Vr
11	Kabul	Afghan Vr	25	Nik Nezhad	Iranian Cv	39	S-B86	Swiss Vr
12	KC2165	Iranian Landrace	26	Orzinal	Iranian Cv	40	ShahPasand	Iranian Cv
13	KC3891	Iranian Landrace	27	Qods	Iranian Cv	41	Shiraz	Iranian Cv
14	KC4144	Iranian Landrace	28	S-B12	Swiss Vr	42	Tschardeh	Iranian Cv

Table 1. Codes and origin of bread wheat genotypes used in current study. Cv: cultivar, Vr. Variety.

of plants in each replication reached physiological maturity. The traits grain number per spike, grain weight per spike, thousand grain weight (g) and grain yield (g/m^2) were measured after harvesting plant.

Grain iron and zinc content measurements

Analysis of grain iron and zinc concentrations was performed by using two staining assays and atomic absorption spectrophotometry method in 42 wheat varieties grown in the field.

Staining method

Perl's Prussian Blue (PPB) staining liquid was used for quantification of the Fe concentration in wheat grains[56]. Treating seeds with PPB causes blue color spots on iron accumulation regions. The intensity of the blue color reaction in the embryo of grains is clearly indicative of grain Fe concentration. The technique did not require the grains to be ground, dry ashed, or digested. PPB solution was prepared by dissolving 60 grams of potassium hexacyanoferrate (II) dihydrate powder in one liter of distilled water. A normal hydrochloric acid (HCl) solution was obtained by adding 125 ml of 32% HCL to one liter of distilled water. In order to determine the location and level of iron concentration in grain, the grains were sanded with a rasp so that the three layers of aleurone, endosperm and embryo were visible. For each genotype, 10 seeds were weighed and fixed by epoxy resin on 3 glass slides. Then the slides were washed with distilled water and placed in PPB reagent solution for 10 minutes to achieve maximum staining. The slides were removed from the reagent and washed again by distilled water and placed under a hood to dry completely. After treating the grains with PPB, the grains were photographed and the pictures analyzed using Adobe Photoshop® software V18.1 (2012). Subsequent image analysis with Adobe Photoshop® to determine pixel numbers in the stained regions lead to the quantification of Fe.

A Zn-chelating agent, Dithizone (DTZ, diphenyl thiocarbazone), has the potential to estimate Zn status in grains. DTZ ($C_6H_5NHNHCSN{=}NC_6H_5$) is soluble in chloroform and gives a dark green color to the solution[57]. When DTZ is added to a solution containing Zn, the color turns red. Dithizone causes red color spots on zinc accumulation regions in grain. Similar to the method of preparing slides for determining grain iron, the slides were dried overnight at room temperature and 500 mg of DTZ powder was dissolved in one liter of pure methanol. Then, the slides were washed with distilled water and placed in DTZ reagent for 30 minutes to achieve maximum staining. After treating seeds with DTZ, the stained sections were photographed and the pictures were analyzed by Adobe Photoshop® software V18.1. For accounting the pixels of each micronutrient in Adobe Photoshop® software, color range option was selected and the color width adjusted at 132 for DTZ and 46 for PPB methods. The number of pixels were counted in color related sections for each staining methods (blue for Fe and red for Zn micronutrients). Then, pixel/mg for Fe and Zn colored sections in grain were obtained by dividing total pixel numbers by seed weight (g).

Quantification of micronutrients by atomic absorption

Quantification of iron and zinc in wheat grains were also performed using AAS equipment in the Department of Soil Science, Shiraz University, Iran. Briefly, the grains were ground and 1 g of the powders were placed in oven at 550 °C for 2 hours. Then, 5 ml of HCl was added to the ash and the samples were passed through filter paper (Whatman No. 1). The final volume was increased to 50 ml by adding distilled water. The Fe and Zn concentration readings were performed using Shimadzu AA-670G instrument.

Polymerase chain reaction (PCR) for zinc and iron related SSR markers

Zn and Fe content related SSR markers were selected from previous studies (Table 2). DNA extraction from leaves were done by CTAB method. The PCR was done using Taq DNA polymerase master mix (AMPLIQON Company) and thermocycler instrument. The PCR program is shown in the Supplementary Table 2.

Marker	Forward/Reverse	Sequence (5'–3')	Annealing temperature (°C)	Amplicon (bp)	References
Xgwm154	Forward	TCACAGAGAGAGAGGGAGGG	59	120	Roder et al. (1998[82])
	Reverse	ATGTGTACATGTTGCCTGCA			
Xgwm192	Forward	GATCTGCTCTACTCTCCTCC	58	136	Roder et al. (1998[82])
	Reverse	CGACGCAGAACTTAAACAAG			
Xgwm538	Forward	GCATTTCGGGTGAACCC	57.5	168	Roder et al. (1998[82])
	Reverse	GTTGCATGTATACGTTAAGCGG			
Xuhw89	Forward	TCTCCAAGAGGGGAGAGACA	61	126	Distelfeld et al. (2006)
	Reverse	TTCCTCTACCCATGAATCTAGCA			

Table 2. Sequence of the primers for the iron and zinc related microsatellite markers.

Statistical analysis and genetic variation parameters

The combined analysis of variance (c-ANOVA), and estimation of genetic variances were performed using the data of the traits measured in the RCBD design for each year. The ANOVA, regression and correlation analysis were performed in the SAS V9.4 software. In group comparison of different commercial cultivars and landraces with respect to traits measured, the *p-values* showing significant differences between groups were obtained based on F-test in ANOVA. Simple correlation coefficients were computed for analysis of simple relation of traits in SAS V9.4 software. The canonical correlation analysis (CCA) performed (in SAS V9.4 software) is a powerful tool that can effectively reduce the dimensionality of data by identifying the most significant linear combinations of variables within each set. The results of CCA are typically straightforward to interpret, as the canonical variables highlight the most correlated pairs of variables between the two sets, showcasing their distinct natures. The NTSYS V2.10, GeneAIEx v.6.5[58], Power marker v3.25[59] and PAST V.3.0.[60] softwares were used for Principal Coordinate Analysis (PCoA), Analysis of Molecular Variance (AMOVA) and Polymorphism Information Content (PIC) calculations.

Heritability in broad sense (h^2_{bs}), phenotypic coefficient of variation (PCV) and genetic coefficient of variation (GCV) were calculated using the Expected Mean Squares (EMS) of the sources of variations in ANOVA (Table 5) formulas as follows,

$$h^2_{\mathrm{bs}} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2/r}$$

$$\mathrm{PCV} = \frac{\sqrt{\sigma_p^2}}{\overline{X}} \times 100$$

$$\mathrm{GCV} = \frac{\sqrt{\sigma_g^2}}{\overline{X}} \times 100$$

where $\overline{X}$, σ_g^2, σ_p^2 and σ_e^2 represent trait mean, genetic, phenotypic and environmental variances, respectively. The EMS of the sources of variations in ANOVA used for estimation of variance componenets were as folllow,

$$\mathrm{EMS}\ \ (\mathrm{phenotype}) = \sigma_e^2 + r\sigma_g^2$$

$$\mathrm{EMS}\ \ (\mathrm{error}) = \sigma_e^2$$

Power Marker v3.25 software was used for calculating PIC for each primer tested in PCR as follows,

$$\mathrm{PIC} = 1 - \sum_{I-1}^{N} Pi^2$$

where *Pi* and *N* are frequeny of present alleles and number of alleles, respectively[61]. The data scored for the amplified bands by the primers in all genotypes were used for analysis of molecular variance (AMOVA) in GenAlex6.5 software[58,62].

Results

Variation in Fe and Zn concentrations among wheat varieties

The normal distribution test for agronomic and micronutrients in wheat genotypes are presented in Supplementary Figs. 1 and 2. The results of c-ANOVA revealed significant differences among wheat varieties for all agronomic traits with non- significant interactions of genotype and year (Table 3). The traits mean in wheat genotypes are shown in Table 4. The range for coefficient of variation was between 2.357% for days to maturity and 25.91%, for grain yield. The result also showed that Danesh, Kabul and Butshak genotypes had the highest grain number per spike and grain weight per spike. The highest thousand grain weight were found in Danesh,

Source	D.F	Mean squares							
		GWS	GNS	TGW	PH	LMS	SNS	DTM	GY
Year	1	0.204^{ns}	63^{ns}	570.84**	3968.25*	60.035^{ns}	252**	15.75^{ns}	26046^{ns}
Block/Year	4	0.068**	36.79**	10.008**	334.78**	9.293**	0.531^{ns}	257.523**	4447.86**
Genotype	41	0.043**	27.603**	7.737**	1161.56**	20.244**	15.941**	213.017**	3891.99**
Genotype × Year	41	0.001^{ns}	2.084^{ns}	2.721^{ns}	6.27^{ns}	0.011^{ns}	4.37^{ns}	6.173^{ns}	6.34^{ns}
Error	164	0.007	4.005	1.964	26.675	1.139	1.718	19.767	951.46
C.V (%)		13.199	11.456	3.978	5.724	8.986	7.21	2.357	25.91

Table 3. Combined analysis of variances (c-ANOVA) for agronomic traits in 42 wheat varieties in two years. C.V: coefficient of variation, D.F: degree of freedom, GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, PH: plant height, LMS: length of main spike, SNS: spikelet number per spike, DTM: day to maturity, GY: grain yield. ns, *, ** are non-significant, significant at 0.05 and 0.01, respectively.

Nik Nezhad, Butshak, KC4551, S-B21, Iran811 and the lowest in KC4848. Bayat, KC4557, Iran811, Iran906 had the highest plant height whilst the lowest was found for S-B74, Orzinal, and Shiraz. The tallest main spike was found in KC4557, Danesh, Shah Pasand and S-B12. The highest spikelet per spike was found in Danesh and S-B136 and the lowest belonged to Mahdavi and Nik Nezhad. The Orzinal and Tschardeh showed late maturity while S-B15 and S-B86 were early matured. The KC4557 and Danesh had higher grain yield than other genotypes. Analysis of micronutrients in the tested genotypes showed that KC4551, KC2165, S-B79 and S-B120 accumulated higher grain's Fe than others. KC4863, S-B122, Mahdavi and KC4848 were top ranked genotypes for Zn content while the Iran 906, KC4144, S-B29 and S-B136 showed lowest Zn in grains.

Scatterplot of wheat varieties for grain yield and grain micronutrients

The three-dimensional plot for grain yield and iron and zinc contents showed the scatter of genotypes for three traits in wheat (Fig. 1). Among genotypes, the S-B79 variety showed high values for grain yield and Zn and Fe contents. KC4551 and KC4863 showed high Zn and Fe contents but low grain yield. The KC4557 showed high grain yield and Fe content but low Zn. S-B126 and S-B122 were average for grain yield and micronutrients.

Group comparison of diverse wheat varieties for agronomic traits and micronutrients

The results of group comparison among Afghan and Swiss varieties and Iranian commercial cultivars and landraces for agronomic traits and micronutrients are shown in Tables 5 and 6. The results showed significant differences between the four groups for most of agronomic traits and micronutrients. Iranian landraces and commercial cultivars showed significant differences for all traits except thousand grain weight. Almost all traits showed significant difference in landrace versus (vs.) Afghan, Iranian commercial cultivars vs. Swiss vanities, and Iranian commercial cultivars vs. Afghan varieties. Swiss vs. Afghan mean comparisons were not significant for thousand grain weight, spikelet number per spike and grain yield. The group comparison results revealed that the two landrace and commercial groups had higher grain yield and grain Zn concentration than other groups and that landrace and Swiss groups had highest grain Fe concentration.

Genetic variation and heritability of agronomic traits and micronutrients

The potential of the diverse wheat genotypes for the improvement of yield and micronutrients were assessed by estimating genetic variation and heritability of traits. Phenotypic coefficients of variation (PCVs) calculated using variance components were higher than genotypic coefficient of variation (GCVs) for all traits tested (Table 7). The GCV ranged between 13.51 and 33.19% for agronomic traits and between 19.61 and 41.86% for micronutrients (Table 7). The highest GCV was found for Fe and the GCV for Zn was higher than other traits except grain yield. The heritability of traits varied between 53% for spikelet number per spike and 79.43% for spike length. Heritability of micronutrients was higher than all agronomic traits except days to maturity and spike length.

Simple and canonical correlations between agronomic traits and micronutrients

The results of simple correlation analysis indicated that correlations between yield and grain weight per spike ($r = 0.46$) and between grain number per spike ($r = 0.505$) were significant (Fig. 2). Grain number per spike was highly correlated with grain weight per spike ($r = 0.954$). Grain weight per spike showed moderate correlation with thousand grain weight ($r = 0.407$). Iron content showed positive correlations with Zn (for both staining and absorption methods) and days to maturity ($r = 0.46$ and 0.48). Staining method and AAS showed strong and significant correlations ($r > 0.90$) for both Fe and Zn contents (Supplementary Table 3). Results of CCA for the two sets of agronomic and micronutrient traits revealed that Zn in wheat grain directly associated with agronomic traits including yield related traits (Fig. 3). However, Fe content had no associations with yield and yield components. The CCA indicated that micronutrients had weak associations with days to maturity.

Cluster analysis of wheat varieties for agronomic traits micronutrients and SSR markers

The cluster analysis divided the 42 wheat genotypes into 3 main groups based on similarity of genotypes for agronomic traits and micronutrients (Fig. 4). Differences of three identified groups are shown in Table 8. The

Genotype code	GWS	GNS	TGW	PH	SL	SN	DTM	YLD	DTZ Fe	DTZ Zn	Zn-Ab	Fe-Ab
1	0.68	19.17	34.37	78.08	11.33	17.67	188.50	150.00	1236.33	2332.00	35.77	26.63
2	0.67	18.50	35.14	76.08	12.67	18.33	193.83	124.45	1406.67	2444.67	44.00	29.40
3	0.57	15.83	34.56	69.42	12.83	18.67	197.17	101.54	1421.67	2447.67	46.28	31.35
4	0.68	18.50	35.83	85.58	11.08	18.33	191.50	163.71	1418.00	2499.33	47.53	32.78
5	0.52	14.83	33.94	90.58	14.42	20.67	195.83	106.62	1402.00	2397.00	41.65	31.68
6	0.69	19.50	34.69	77.50	10.08	18.33	192.17	157.50	1333.67	2399.67	40.93	32.75
7	0.60	17.17	34.06	79.67	13.17	20.00	182.50	136.97	1378.67	2525.67	50.05	38.03
8	0.70	19.83	34.34	91.83	13.17	18.00	182.17	121.70	1403.00	2421.00	39.58	37.48
9	0.60	17.50	33.24	74.00	9.75	18.33	183.50	123.63	1510.33	2559.00	55.75	47.00
10	0.45	13.50	31.84	110.83	9.92	15.00	180.83	79.01	1558.67	2497.33	49.77	51.42
11	0.56	15.83	34.38	91.75	11.75	19.67	193.17	138.84	1259.67	2425.00	42.42	26.53
12	0.93	24.83	36.69	78.25	16.50	25.00	191.83	167.91	1397.67	2390.33	39.05	37.17
13	0.62	17.50	34.09	89.67	13.42	18.33	195.83	146.32	1541.33	2509.00	50.95	47.68
14	0.63	17.83	34.45	97.50	11.50	17.67	191.17	109.26	1745.67	2612.00	61.22	63.72
15	0.68	18.83	35.16	107.00	14.08	19.33	191.83	58.98	1661.67	2408.00	40.85	62.90
16	0.59	16.17	35.43	104.33	11.08	17.67	196.50	131.20	1563.67	2289.00	28.93	51.10
17	0.64	17.83	34.32	113.33	13.33	18.67	194.83	169.57	1666.33	2388.00	38.82	59.72
18	0.59	16.50	34.00	94.50	8.42	14.33	181.50	139.52	1749.67	2457.33	45.73	75.77
19	0.50	14.17	33.46	92.17	12.42	17.33	185.83	95.83	1862.67	2349.33	34.97	96.10
20	0.63	16.83	37.14	80.83	13.83	19.33	184.83	142.05	1914.33	2539.33	53.95	102.97
21	0.58	15.50	35.93	78.00	14.42	20.00	186.50	119.11	1753.00	2402.33	40.25	73.68
22	0.56	15.50	34.85	69.83	13.42	18.33	187.17	117.62	1780.67	2498.00	49.83	77.52
23	0.67	18.17	36.04	75.50	11.50	17.33	185.50	127.33	1791.00	2436.67	43.68	75.60
24	0.58	16.17	34.72	75.00	12.42	17.67	180.50	111.08	1545.00	2348.33	34.87	50.52
25	0.64	16.83	36.57	81.50	11.17	17.33	178.83	92.66	1538.33	2394.67	39.50	51.48
26	0.68	18.17	36.44	71.83	10.92	18.33	184.50	122.82	1585.67	2334.00	33.40	53.78
27	0.60	16.17	35.93	76.67	12.50	18.67	182.17	94.85	1545.00	2401.67	40.17	51.65
28	0.59	16.17	35.57	78.50	11.50	16.67	182.83	121.51	1554.00	2377.33	37.75	54.72
29	0.66	17.50	36.39	76.75	11.25	17.00	177.50	103.09	1736.00	2436.00	43.65	71.28
30	0.56	14.83	36.19	89.33	12.00	17.33	179.17	87.28	1603.00	2397.67	39.80	58.88
31	0.66	17.83	35.95	70.33	12.08	18.33	188.83	97.70	1522.33	2558.00	55.83	52.07
32	0.62	16.83	35.89	77.42	11.83	18.33	183.50	116.38	1566.67	2523.33	52.35	54.60
33	0.70	18.17	37.31	116.83	10.75	18.67	193.83	139.00	1571.33	2453.00	45.22	57.05
34	0.53	14.83	34.48	77.58	10.83	17.67	188.17	121.10	1381.33	2539.67	54.00	40.88
35	0.77	20.50	36.74	105.67	11.50	18.33	187.17	102.05	1312.67	2482.33	48.12	38.03
36	0.69	18.83	35.51	106.17	9.92	15.33	190.83	143.13	1313.67	2387.33	38.77	38.95
37	0.61	16.50	35.64	116.83	13.42	18.00	187.83	121.81	1212.67	2249.67	24.98	27.47
38	0.60	16.50	35.47	75.67	12.33	17.33	194.50	76.01	1277.67	2354.67	35.50	35.75
39	0.83	22.83	35.44	96.67	7.75	17.00	195.50	140.45	1283.00	2454.67	45.48	32.93
40	0.66	17.50	36.41	88.33	6.75	18.00	198.17	128.56	1206.67	2382.33	38.25	26.07
41	0.67	18.17	35.66	66.42	10.08	18.67	198.17	84.77	1206.67	2396.00	39.47	26.63

Continued

Genotype code	GWS	GNS	TGW	PH	SL	SN	DTM	YLD	DTZ Fe	DTZ Zn	Zn-Ab	Fe-Ab
42	0.71	19.50	35.42	87.67	13.08	18.67	194.50	101.19	1186.00	2326.33	32.67	26.65
LSD (0.05)*	0.09	2.28	1.60	5.90	1.21	1.49	5.07	35.16	187.9	110.03	9.95	21.25

Table 4. Means for agronomic traits and micronutrients in 42 wheat genotypes. GWS: Grain weight of seeds, GNS: Grain number of spike, LSD: least significant differences, TGW: thousand grain weight, PH: plant's height, SL: Spike length, SN: spike number, DTM: day to maturity, YLD: yield, DTZ Fe: Fe content measured by DTZ staining method, DTZ Zn: Zn content measured by DTZ staining method, Fe-Ab: Fe content measured by atomic absorption spectrophotometer, Zn-ab: Zn content measured by atomic absorption spectrophotometer,* means with differences higher than LSD value are significant in each column.

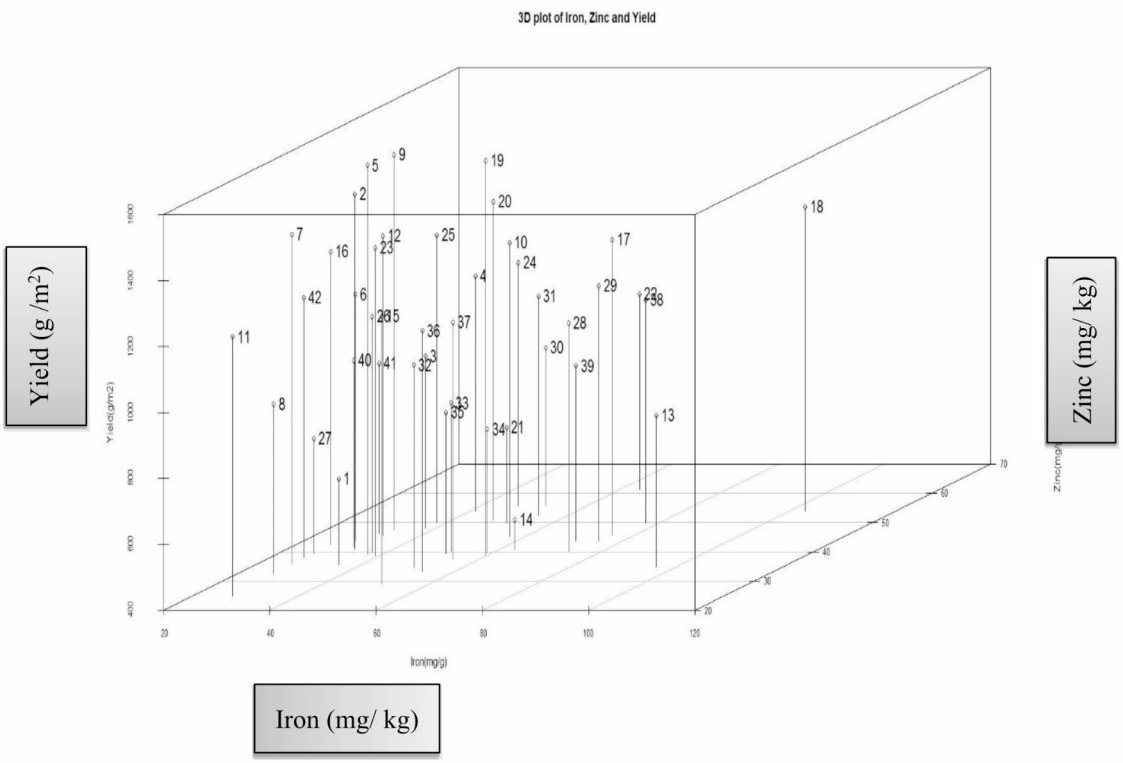

Fig. 1. Three-dimensional diagram showing scatter of wheat varieties for grain yield, iron and zinc contents. The numbers stand for the code of genotypes in Table 1.

Traits	Mean								
	Landraces	Swiss	P-value	Landraces	Commercial cultivars	P-value	Landraces	Afghan	P-value
GWS (g)	0.56	0.6	*0.001*	0.56	0.62	*0.001*	0.56	0.65	*0.01*
GNS	16.0	16.0	*0.38*	16.0	18.0	*0.0008*	16.0	18.0	*0.0008*
TGW (g)	32.76	34.71	*0.01*	32.76	32.61	*0.02*	32.76	34.62	*0.001*
SL (cm)	11.0	12.0	*0.0001*	11.0	12.0	*0.0001*	11.0	10.0	*0.0001*
PH (cm)	101	79.67	*0.0001*	101	83.67	*0.0001*	101	96.73	*0.002*
SNS	17.0	17.0	*0.03*	17.0	18.0	*0.0001*	17.0	17.0	*0.02*
DTM	187	181	*0.0002*	187	187	*0.01*	187.0	190.0	*0.0001*
YLD (g/m^2)	111	99.2	*0.11*	111	125.4	*0.003*	111.0	106.5	*0.001*
Fe-DTZ (pix/g)	1636	1626	*0.0001*	1636	1406	*0.0001*	1636	1295	*0.0001*
Zn-DTZ (pix/g)	2436	2425	*0.0001*	2436	2452	*0.0003*	2436	2405	*0.0001*
Fe-ab (mg/kg)	63.79	60.48	*0.0008*	63.79	34.43	*0.0001*	63.79	35.04	*0.0001*
Zn-ab (mg/kg)	44.76	42.59	*0.0001*	44.76	44.06	*0.002*	44.76	40.25	*0.0001*

Table 5. Comparison agronomic traits and Zn and Fe concentrations in four groups of Afghan and Swiss varieties and Iranian commercial cultivars and landraces. GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, SL: spike length, PH: plant height, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield, Fe-PPB: Fe content measurement by Pearl's Prussian Blue method, Zn-DTZ: Zn content measurement by DTZ method, Fe-ab: Fe content measurement by atomic absorption spectrophotometer, Zn-ab: Zn content measurement by atomic absorption spectrophotometer.

Traits	Mean								
	Commercial cultivars	Swiss	P-value	Commercial cultivars	Afghan	P-value	Swiss	Afghan	P-value
GWS (g)	0.62	0.6	*0.0009*	0.62	0.65	*0.001*	0.6	0.65	*0.10*
GNS	18.0	16.0	*0.0001*	18.0	18.0	*0.20*	16.0	18.0	*0.0001*
TGW (g)	32.61	34.71	*0.006*	32.61	34.62	*0.005*	34.71	34.62	*0.12*
SL (cm)	12.0	12.0	*0.0001*	12.0	10.0	*0.0001*	12	10	*0.0001*
PH (cm)	83.67	79.61	*0.002*	83.67	96.73	*0.001*	79.61	96.73	*0.001*
SNS	18.0	17.0	*0.0001*	18.0	17.0	*0.0001*	17.0	17	*0.40*
DTM	187.0	181.0	*0.0004*	187.0	190	*0.0001*	181	190	*0.0001*
YLD (g/m^2)	125.4	99.2	*0.0001*	125.4	106.5	*0.001*	99.2	106.5	*0.20*
Fe-PPB (pix/g)	1406	1626	*0.0001*	1406	1295	*0.0001*	1626	1295	*0.0001*
Zn-DTZ (pix/g)	2452	2425	*0.013*	2452	2405	*0.0001*	2425	2405	*0.019*
Fe-ab (mg/kg)	34.43	60.48	*0.0001*	34.43	35.04	*0.03*	60.48	35.04	*0.0002*
Zn-ab (mg/kg)	44.06	42.59	*0.003*	44.06	40.25	*0.002*	42.59	40.25	*0.004*

Table 6. Comparison agronomic traits and Zn and Fe concentrations in Afghan and, Swiss varieties and Iranian commercial cultivars. GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, SL: spike length, PH: plant height, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield, Fe-PPB: Fe content measurement by Pearl's Prussian Blue method, Zn-DTZ: Zn content measurement by DTZ method, Fe-ab: Fe content measurement by atomic absorption spectrophotometer, Zn-ab: Zn content measurement by atomic absorption spectrophotometer.

Trait	V_G	V_E	V_P	PCV (%)	GCV (%)	h^2
GWS	0.0063	0.005	0.0113	17.43	13.51	55.75
GNS	4.066	3.204	7.271	16	11.96	55.92
TGW	3.856	2.424	6.28	7.43	5.82	61.4
PH	147.193	119.01	266.203	18.17	13.51	55.29
SL	3.526	0.913	4.439	18.69	16.66	79.43
SNS	2.96	2.624	5.584	13.73	10	53.0
DTM	38.139	15.814	53.953	3.98	3.34	70.69
YLD	1334.18	762.43	2096.61	41.61	33.19	63.63
Fe	420.94	171.14	592.08	49.65	41.86	71.1
Zn	71.172	37.51	108.68	24.3	19.67	65.48

Table 7. Variances and broad-sense heritability for agronomic traits and micronutrients. GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, SL: spike length, PH: plant height, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield, V_G: genetic variance, V_E: expected error variance, V_P: phenotypic variance, PCV and GCV: phenotypic and genotypic coefficients variance, h^2, broad-sense heritability.

group 1 had the highest grain weight per spike and thousand grain weight while group 2 showed higher means for Zn concentration, grain number per spike, plant height, length of main spike, spikelet number per spike, day to maturity and yield gain. The mean of Fe concentration was highest in group 3 and the grain yield was the lowest. The commercial cultivars group had relatively high Zn concentration, low Fe concentration and high grain yield. Swiss and landraces varieties showed low yield but high Zn and Fe concentrations. Afghan varieties showed moderate yield and low Zn and Fe concentrations.

The results of cluster analysis using polymorphic SSR markers and UPGMA method are presented in Fig. 5. The results placed 42 genotypes into 4 distinct groups. The group-1 comprised of landrace varieties, whilst all Iranian commercial varieties placed in group-4. The group-2 and group-3 almost showed a mixture of Swiss and Afghan varieties. The results showed that except "Kaboul", all genotypes originated from same country grouped in same group.

Polymorphism information content (PIC) and analysis of molecular variance (AMOVA)

Analysis of PIC for the tested primers showed that primers had various values for PIC (Table 9). The mean PIC was 0.75 with the *Xgwm192* primer presented higher PIC (0.82) than others in the tested wheat varieties (Table 8). Results of AMOVA showed that within population variance had higher contribution (60%) to the total genetic diversity than between population component (Supplementary Table 4).

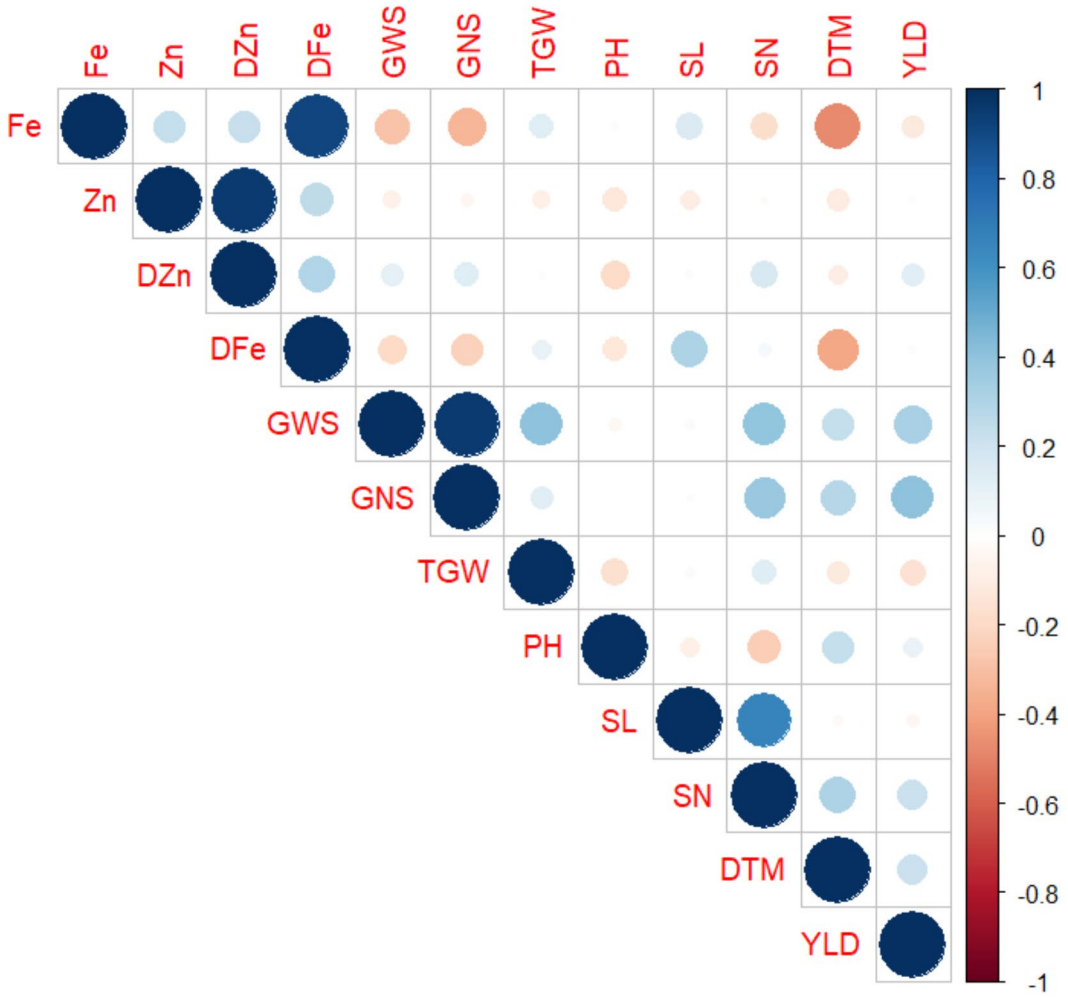

Fig. 2. Simple correlation coefficients among agronomic traits and micronutrients. GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, SL: spike length, PH: plant height, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield, Fe-PPB: Fe content measurement by Pearl's Prussian Blue method, Zn-DTZ: Zn content measurement by DTZ method, Fe-ab: Fe content measurement by atomic absorption method, Zn-ab: Zn content measurement by atomic absorption method.

Principal coordinate analysis (PCoA) for Zn and Fe related markers

The PCoA was used for map-based visualization of association of the SSR markers and wheat genotypes for Fe and Zn contents (Fig. 6 and Supplementary Figs. 3-9). The PCoA of *Xgwm192* marker, differentiated 7 high Fe content genotypes from 10 genotypes with low Fe (Fig. 4). Similar results showed differentiation of high/low Zn genotypes in the PCoA for *Xgwm192* (Supplementary Fig. 3). For Zn content, the high Zn genotypes scattered in same PCoA sector differentiated form low Zn genotypes (Supplementary Fig. 3). The results of PCoA using *Xuhw89* polymorphism in wheat varieties showed that KC4551, KC4848, KC2165, KC454, S-B120, S-B12, S-B86 and S-B79 with high Fe content placed in same sector in the PCoA biplot while Tschardeh, Falat, Forno, Orzinal, Dez, KC4537, Iran906, Kabul and Arina with low Fe content clustered in another sector (Supplementary Fig. 4). The PCoA results for the scattering of genotypes based on the *Xuhw89* and Zn content was consistent with results of PCoA for Fe in wheat varieties (Supplementary Fig. 5). For Zn content, KC4863, S-B122, KC4551, S-B126, Camedo, Madavi and KC4633 placed in common sector identified as high Zn content genotypes while Iran906, KC4144, Forno and S-B29 were low Zn content genotypes. The markers *Xuhw89* and the *Xgwm154* showed similar variety scattering in PCoA with respect to Fe and Zn (Supplementary Figs. 4, 5, 6 and 7). In *Xgwm154* PCoA biplot, KC4551, KC2165, S-B79, KC4542, S-B120, S-B12 and S-B86 known as high Fe content genotypes joint in same sector in PCoA biplot whilst Tschardeh, Falat, Orzinal, Forno, Iran906, Kabul, Arina, Dez، Shiraz and Bayat detected as low Fe content genotypes. Both *Xuhw89* and the *Xgwm154* markers differentiated high

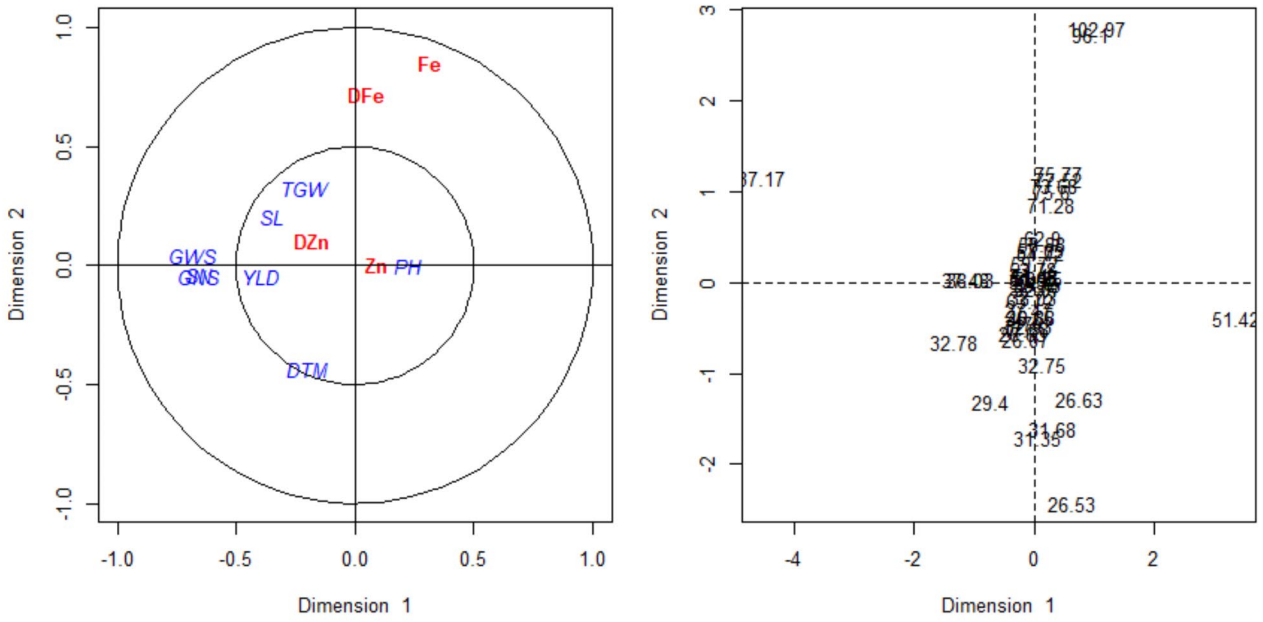

Fig. 3. Canonical correlation analysis for the two sets of agronomic and micronutrient traits tested in 42 wheat varieties. GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, SL: spike length, PH: plant height, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield, Fe-PPB: Fe content measurement by Pearl's Prussian Blue method, Zn-DTZ: Zn content measurement by DTZ method, Fe-ab: Fe content measurement by atomic absorption method, Zn-ab: Zn content measurement by atomic absorption method.

and low Zn content genotypes. For *Xgwm538* marker, genotypes with similar Fe and Zn contents placed in same sector in the PCoA biplot (Supplementary Figs. 8 and 9).

Discussion

Breeding wheat for the accumulation of micronutrients is a sustainable and cost-effective strategy. However, many breeding programs prioritize higher grain yield over the nutritional content of the crop plants[19,63]. Additionally, the lack of user-friendly methods for quantifying micronutrients and the need for analyzing large sample collections hinder efforts to breed for high levels of these essential nutrients in plants. In our study, we assessed the genetic diversity of a core collection of wheat genotypes to determine their variability in micronutrient content and how it relates to agronomic and yield traits. We also evaluated a rapid staining method for localizing iron (Fe) and zinc (Zn) in a large sample size as an efficient alternative to the traditional atomic absorption method for measuring micronutrients. Accessing an efficient quantifying method is essential for plant breeders when dealing with large sample sizes required for measuring variables in breeding programs. Rapid screening methods for evaluating genetic biodiversity of micronutrients not only save time and money but also enable research to be conducted in remote locations with limited access to modern facilities. In our study, staining methods and AAS equipment showed strong correlation (r^2 0.90) for micronutrient levels suggesting the staining method could serve as a reliable alternative to AAS. Furthermore, Ozturk et al.[19] validated the results of micronutrient quantification using the staining method and analyzed the correlation between DTZ staining and spectral absorbance of flour extracts in wheat. The DTZ staining method was utilized to differentiate between high and low zinc wheat varieties in the study conducted by Shariatipour et al.[37].

Our research involved the examination of a diverse core collection of wheat genotypes sourced from various regions worldwide to assess micronutrient biodiversity. The average zinc and iron concentrations in the grains

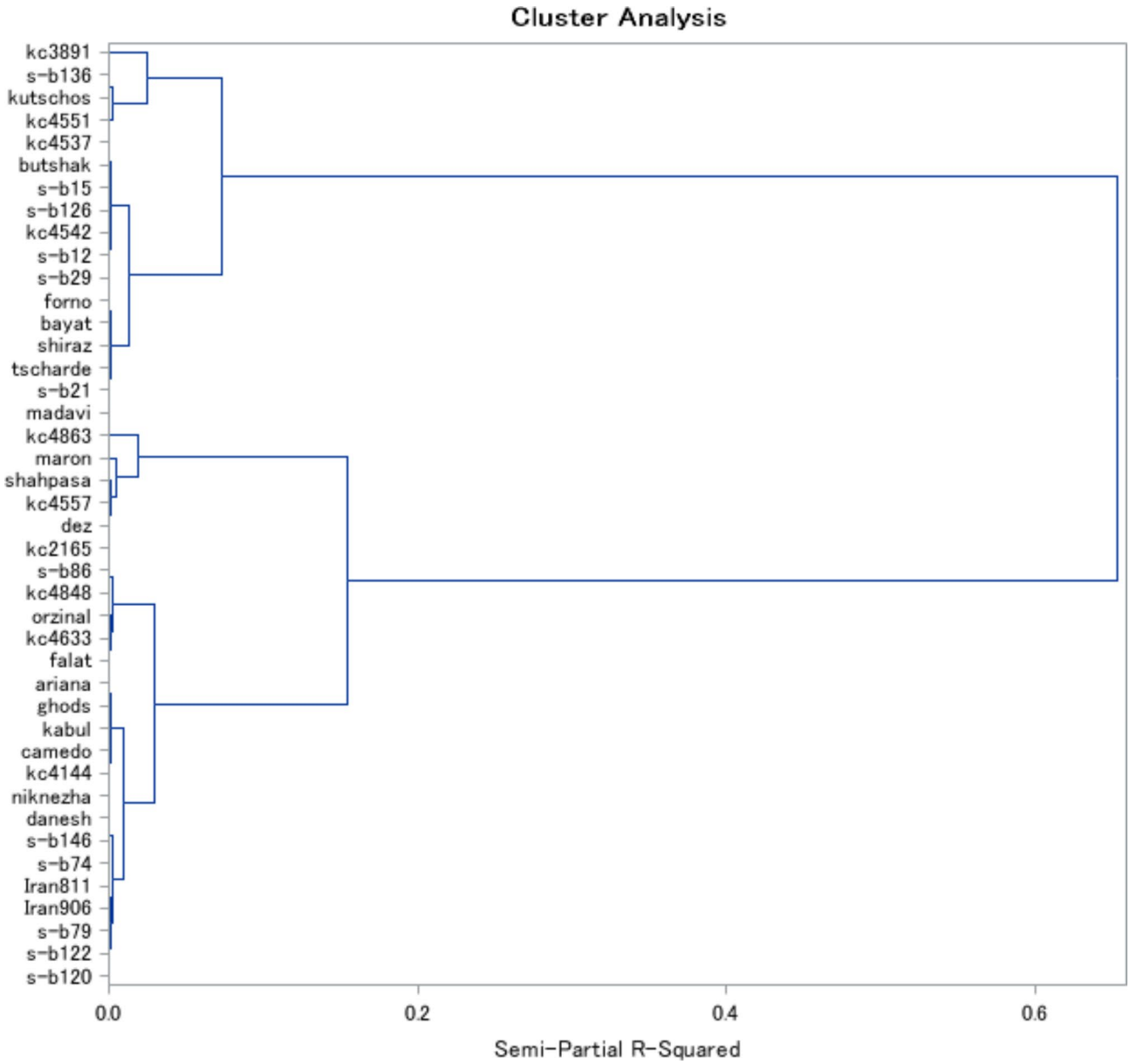

Fig. 4. The tree dendrogram showing 42 varieties divided into three main groups for agronomic traits and micronutrients.

of 42 wheat genotypes in our study exceeded those reported in previous wheat studies[64–68]. The elevated levels of micronutrients identified in our study underscore the significance of genetic diversity in wheat for micronutrient content and highlight the potential of the landraces examined for agronomic traits and micronutrients. Our findings revealed that the average iron concentration in landraces was 53.97% greater than in commercial cultivars. Additionally, the zinc concentration in Iranian landraces was 8.99% higher than in Afghan varieties. However, Swiss varieties exhibited no significant differences in zinc and iron concentrations compared to landraces. Landraces carry valuable genes and show high genetic variation for different traits which can be involved in breeding programs through cross hybridization with commercial cultivars with respect to breeder targeted traits[11,49,50,67,69,70].

The modest negative correlation between grain yield and micronutrients in modern cultivars presents a challenge in breeding for plants that are both high-yielding and rich in micronutrients[68,71–73]. In our study negative correlations were found between Fe concentration and agronomic traits including grain weight, number per spike, days to maturity. Similar findings have been reported in previous studies, demonstrating a negative association between yield components and micronutrient levels in wheat[65,69,71,74]. Simple correlations show relation of two variables without taking into account the effects of other variables whilst CCA considers linear combination of all variable tested for analysis of interrelationship between variables. CCA allows for the exploration of multidimensional relationships between two sets of variables that cannot be captured by simple correlation analysis. Our results from CCA indicated that grain number per spike and thousand grain weight

Traits	Mean			P-value
	Group 1	Group 2	Group 3	
Fe-ab (mg/kg)	38.20	79.58	44.34	*0.0001*
Zn-ab (mg/kg)	40.51	46.66	43.07	*0.195*
GWS (g)	0.64	0.57	0.60	*0.085*
GNS	18.10	16	16.57	*0.041*
TGW (g)	33.51	33.63	33.91	*0.692*
PH (cm)	96.59	86.42	86.34	*0.041*
SL (cm)	10.89	11.56	11.41	*0.657*
SNS	17.62	16.67	17.08	*0.399*
DTM	189.86	182.50	184.9	*0.007*
YLD (g/m^2)	135.85	109.23	92.27	*0.0001*

Table 8. Differences of three main groups of wheat varieties identified in cluster analysis for agronomic traits and micronutrients. Fe-ab: Iron measured by atomic absorption spectrophotometer, Zn-ab: Zinc measured by atomic absorption by spectrophotometer, GWS: grain weight per spike, GNS: grain number per spike, TGW: thousand grain weight, PH: plant height, SL: spike length, SNS: spikelet number per spike, DTM: day to maturity, YLD: grain yield.

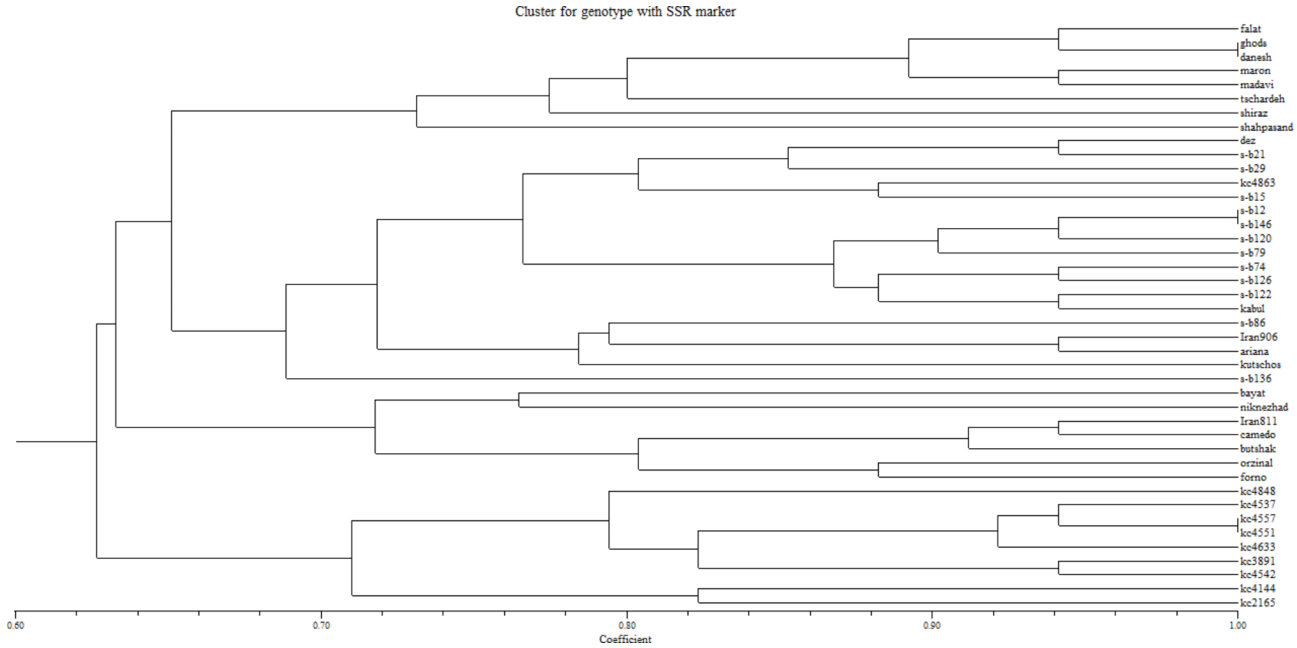

Fig. 5. The tree dendrogram of 42 wheat genotypes grouped using simple sequence repeats (SSR) polymorphic data.

Marker	Genetic variation	Polymorphism information content (PIC)
Xuhw89	0.77	0.74
Xgwm154	0.79	0.76
Xgwm192	0.84	0.82
Xgwm538	0.73	0.70
Mean	0.78	0.75

Table 9. Genetic variation and polymorphism information content (PIC) of four SSR markers tested in 42 wheat genotypes.

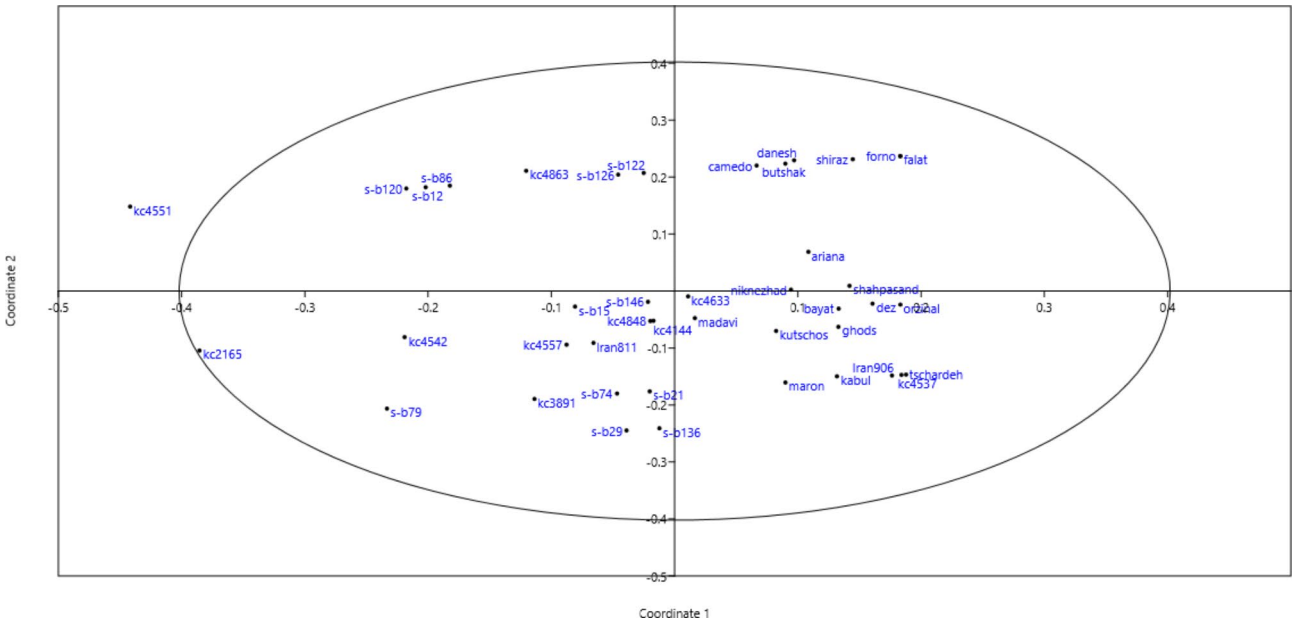

Fig. 6. The principal co-ordinates analysis (PCoA) of wheat varieties as revealed differentiation of high/low micronutrient varieties by the *Xgwm192* marker. Codes stand for the wheat varieties as shown in Table 1.

exhibited stronger associations with grain Fe content, consistent with findings from a study by Marcos-Barbero et al.[75] in wheat. However, grain number showed a negative association with Fe, Zn, and Mg[75]. The correlation of nutrients with growth related traits might be influenced by physiological associations in plant and soil. The main component controlling the availability of metallic micronutrients are carbonates that are dominant in the soils of arid and semiarid regions. Carbonates adsorb/precipitate the metallic micronutrients. The metallic micronutrient especially iron reacted with carbonate or bicarbonate and produces the metal-carbonate in which the metal is not available for plants. High amount of calcium carbonate in soils in Iran, low amount of organic matter, and alkaline pH affect the availability of nutrients to plants and usually lead to deficiency for the micronutrients[76]. It has been shown that environmental factors, source-sink interactions, and dilution of micronutrients by non-micronutrient compounds are the major agents that caused undesirable associations between the grain yield and micronutrients traits[77]. In a separate study on wheat, CCA and the DTZ staining method revealed a positive and significant correlation between Zn content and grain yield[37]. The inconsistent relationships observed between micronutrients and yield traits across different studies demonstrated that interaction of micronutrients and growth-related traits should be considered in breeding programs and have implications for breeding efforts aimed at developing high-yielding, high-quality wheat varieties.

The success of wheat breeding is heavily dependent on the level of heritability and the availability of genetic variation for the desired trait, both of which necessitate a wide range of germplasm resources. Heritability refers to the extent to which genetic factors contribute to variations in traits. Our findings indicate that the genotypic coefficient of variation, which reflects the degree of genetic variability, was higher for micronutrients compared to most agronomic traits in our wheat germplasm. Similarly, the heritability of micronutrients was found to be greater than that of agronomic traits, with the exceptions of days to maturity and spike length. The heritability of micronutrients (iron and zinc), in grains was over 60%, indicating a significant genetic influence on their phenotypic variation. This suggests that genetic factors play a larger role than environmental components in determining the levels of iron and zinc in wheat grains. This high heritability accompanied with high genetic variation observed imply high response to selection and that breeding programs can effectively select for improved wheat quality by focusing on additive genetic components related to these essential nutrients. In a study by Zecevic et al.[78], it was observed that the heritability of grain weight and number per spike in wheat was comparable to heritabilities of other trait. These results align with the findings of Heidari et al.[50], who also reported similar genotypic coefficient of variation values and heritability estimates for micronutrients in wheat. Additionally, Ozturk et al.[19] discovered that there was significant genetic variability for grain zinc (Zn) and iron (Fe) concentrations in Mexican germplasm, suggesting the potential for selecting cultivars with high yields and concentrations of these essential nutrients.

The current neutral theory has not fully grasped the concept of genetic diversity[79]. Recent research has revealed that short tandem repeats in DNA play a crucial role in regulating levels of transcription factor binding and subsequent gene expression[80]. While SSR repeats were previously thought to be neutral, multiple studies have suggested that these markers can actually impact transcription by directly influencing the affinity of histone proteins for DNA, leading to changes in nucleosome occupancy[79,80]. Our study indicated that information obtained from polymorphic SSR markers are instrumental in distinguishing between high and low micronutrient varieties through a Principal Coordinates Analysis (PCoA). In the PCoA of our study, the *Xuhw89* marker emerged as a key contributor to the total variance of the first PCoA. The results of the PCoA

clearly demonstrated the ability of all SSR markers to effectively differentiate between genotypes with varying micronutrient levels. This underscores the advantages of utilizing linked markers for the rapid screening of a wide range of wheat varieties in the early stages of growth, particularly for micronutrient content and in the context of speed breeding programs aimed at enhancing the quality of wheat cultivars. Functional nature of SSR markers has been reported in Lebedev et al.[81] study, demonstrating high polymorphism of the SSR markers in regulatory flavonoid biosynthesis genes which suggests their allelic variability that can be potentially associated with differences in flavonoid accumulation and composition. The utilization of marker technology has paved the way for the development of speed breeding and marker-assisted selection (MAS) programs. In our study, an analysis of molecular variance (AMOVA) using polymorphic SSR markers has revealed significant levels of genetic diversity both within and among populations, providing valuable insights for use of SSR markers in the selection of genotypes with high Zn and Fe content. High heritability identified for micronutrients in this study demonstrates that application of SSR markers helps breeders for increasing selection efficiency of high micronutrients plants in the early stages of growth without the need for use of destructive methods for measuring grain micronutrients and saves the grains for further breeding programs.

Conclusions

This study provided valuable insights in three key research areas with respect to the genetic diversity assessment and the improvement of micronutrients in wheat breeding programs. First, the strong and significant correlations observed between the micronutrients data obtained in staining method and atomic absorption equipment demonstrated that staining methods are reliable, user friendly and cheaper options for quantification of mineral in breeding programs where the cost and access to atomic absorption facility is limited for screening large samples. Secondly, the tested germplasm displayed significant genetic diversity for micronutrients, identified through both biometrical analysis of phenotypic data and SSR polymorphic markers, with landraces exhibiting higher levels of Fe and Zn compared to the commercial cultivars. This emphasizes the importance of incorporating landraces and use of their useful genes in breeding programs aimed at enhancing micronutrient content in wheat. Lastly, the study emphasizes the identified SSR markers linked with micronutrients can be applied for screening large sample size populations for micronutrient content and allows identifying superior plants at the early stages of growth and high selection efficiency in marker-assisted selection (MAS) programs aimed at development of biofortified (iron and zinc) wheat grains.

Data availability

Data availability: No sequencing data was used in the current study and all the datasets are presented in the article/supplementary files.

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Author contributions

B.H. designed the research and the experiments, supervised the project, prepared samples, wrote and reviewed the initial and final drafts of the manuscript, D.B. performed the experiments, prepared samples, and analyzed the data, T.M.M. contributed to writing the manuscript and re-analyzing the data, V.G. provided scientific comments and reviewed the final draft of the manuscript. All authors read and approved the final draft of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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