

Analysis of Diversity And Identification of SSR, SCoT, And ITAP Informative Amplicons For Grain Fe And Zn Content In Wheat Genotypes

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Abstract

Biofortification provides a promising method of solving microelement malnutrition in developing countries. For this purpose, a study was conducted to understand the grain Fe and Zn content variation in seventy prevalent Iranian wheat genotypes across three consecutive years, to assess genetic diversity, and to identify informative amplicons for high grain Fe and Zn content using three simple sequence repeat (SSR), start codon targeted (SCoT) polymorphism, and intron targeted amplified polymorphism (ITAP) markers. Grain Fe and Zn content was highly variable each year with high heritability. Despite the highly significant effect of year-genotype interaction, some stable genotypes were ranked highly all the three years for grain Fe and Zn content. The grain Fe and Zn contents were positively correlated in the second and third years. High genetic diversity was detected among the wheat genotypes using three different marker systems. A number of informative SSR, SCoT, and ITAP amplicons for high grain Fe and Zn were identified overall or in individual years. A few informative amplicons were common and stable for grain Fe and Zn content in the different years. The SSR alleles located on 3A, 4A, 4B, and 6B chromosomes were positively correlated with high Fe and Zn content, indicating that co-location of genes affected Fe and Zn content. Identification of informative alleles and amplicons for high grain Fe and Zn content could contribute to the development of sequence-based markers and improve the selection of genotypes with high micronutrient content.

Introduction

As a crop grown worldwide, bread wheat (*Triticum aestivum* L.) is a crucial source of nutrition, and supplies over 20% of dietary protein and food calories (FAOSTAT 2018; Trkulja et al. 2019; Phuke et al. 2020). Although cereal crops make up a significant part of people's dietary intake around the world, the mineral micronutrient content of cereal grains, including Fe and Zn, is generally inadequate and low (Borrill et al. 2014; Srinivasa et al. 2014). Minerals are essential elements in the vital functions of the human body. Additionally, micronutrients stored in grains of staple food crops are critical factors to increase plant productivity and resistance against biotic and abiotic stress in plants, mainly when planted on poor soils (Welch and Graham 2005; Palmer et al. 2014). Malnutrition of minerals such as Fe and Zn is a significant health problem in developing countries that rely on cereals as a vital staple food, leading to illness and even death (Welch and Graham 2005; Srinivasa et al. 2014). Many strategies, including fortification, supplementation, and use of diverse food sources, have been suggested to compensate for deficiencies. For technical and economic reasons, however, these strategies are not entirely efficient and sustainable, especially for the poor society sector of the world's population (Borrill et al. 2014; Saini et al. 2020). Root absorption, translocation, and accumulation of mineral microelements in edible portions of seeds and grains are under genetic control (Amiri et al. 2015; Saini et al. 2020). Nevertheless, studies indicate that environmental factors and their interaction with genetic factors play an effective role in specification of the grain mineral micronutrient contents of plants (Cakmak et al. 2004; Amiri et al. 2015). Biofortification denotes genetic improvement of micronutrient bioavailability, and grain micronutrient content control is an efficient, secure strategy to resolve the problem of micronutrient deficiency in major foods (Welch and Graham 2005; Borrill et al. 2014; Kumar et al. 2018a, b). Research findings show that there is substantial diversity for minerals within the wheat populations that provide the potential for breeding programs (Welch and Graham 2005; Badakhshan et al. 2013; Pandey et al. 2016). This variation could be caused by the different potentials of genotypes in root absorption of micronutrients from the soil or micronutrient saving in the edible portions of grains, providing a screening and selection of genotypes with high microelement content for biofortification through either conventional and molecular breeding or genetic engineering (Borrill et al. 2014; Amiri et al. 2015; Kumar et al. 2018b). Genetic diversity for grain micronutrients is crucial for successful biofortification, identification, and selection of future donors (Srinivasa et al. 2014). Genetic diversity is fundamental to conventional and molecular plant breeding strategies such as marker-assisted selection, quantitative trait loci (QTL) mapping, and genomic selection (Eltaher et al. 2018). Several DNA markers have been used widely in genetic studies due to simplicity, informativeness, and cost-effectiveness (Xiong et al. 2013; Eltaher et al. 2018; Rufo et al. 2019). However, simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers target specific sequence regions, and function as codominant markers. Therefore, these markers have been used widely for genetic diversity analysis, cultivar identification, association, and phylogenetic analysis (Yang et al. 2016; Eltaher et al. 2018). Several novel techniques of gene-targeted molecular marker techniques have also been innovated recently, such as start codon targeted (SCoT) and intron targeted amplified polymorphism (ITAP), acting as semi-specific and semi-functional and revealing polymorphism ideally in the plant kingdom (Xiong et al. 2013). The SSR and SCoT markers have been used in several previous studies for investigation of genetic diversity in wheat populations (Naghavi et al. 2009; Senturk Akfirat and Uncuoglu 2013; Nasrollahi et al. 2019; Khodaei et al. 2021). Based on our knowledge, however, there is no report on employment of ITAP markers in genetic studies of wheat. Informative markers (*i.e.* correlated molecular markers with phenotypic traits) make it possible to select reliable genotypes. Marker-assisted selection (MAS) is a highly efficient, dependable method in plant breeding programs.

The objectives of this research are (i) to investigate variation and genotype-environment interaction in wheat grain Fe and Zn content, (ii) characterize the genetic diversity and population structures of the wheat genotypes prevalent in Iran using SSR, SCoT, and ITAP markers, and (iii) to specify informative SSR, SCoT, and ITAP amplicons for grain Fe and Zn contents.

Materials And Methods

A set of sixty-seven bread and three durum wheat genotypes obtained from Seed and Plant Improvement Institute (SPII) in Karaj, Iran were planted for three years (2017–2019) in a loam-clay soil at the research field of the University of Kurdistan. A list of the studied wheat genotypes is presented in Table 1 along with pedigree information. Each genotype was grown in a plot consisting of three rows with 0.2 m distances based on randomized complete block design (RCBD) with three replications. Before sowing, samples were collected randomly from the top 0–30 cm of the soil at the experimental site and subsequently analyzed for micronutrients, organic matter, and pH (Supplementary Table S1). The wheat genotypes were assessed for grain Fe and Zn content. The genetic diversity of the wheat genotypes was also analyzed using SCoT, ITAP, and SSR primers. The Chinese Spring cultivar was used as a reference for examination of SSR primers.

Table 1- Studied wheat genotypes, pedigree, growth habit, and origin

Wheat Genotypes	Pedigree	Growth Habit	Origin
Golestan	KAZ/(SIB)BUHO//KALYANSONA/BLUEBIRD	Spring	CYMMIT
Chamran	NORD-DESPREZ/VG-9144//KAL/BLUEBIRD/3/(SIB)YACO/4/(SIB)VEERY-5	Spring	CYMMIT
Tajan	BOBWHITE/NEELKANT	Spring	CYMMIT
Star	LFN/SDY//PVN	Spring	CYMMIT
Shiraz	GAVILAN, MEX/D-6301//((SIB)ALONDRA/3/AZADI	Spring	CYMMIT
Roshan	Landrace	Spring	IRAN
Alamout	KVZ/TI71/3/MAYA'S//BB/INIA/4/KARAJ2/5/ANZA/PI/NAR//HYS	Spring	CYMMIT
CrossShahi		Spring	IRAN
Arvand	ROSHAN/3/MENTANA//KENYA/MAYO-58; RUSHMORE//LERMA/MAYO	Facultative	CYMMIT
Moghan2	LERMA-ROJO-64(SIB)/HUAMANTLA-ROJO	Spring	CYMMIT
Inia	LERMA-ROJO-64/SONORA-64	Spring	CYMMIT
Marvdasht	HD-2172/BLOUDAN//AZADI	Facultative	IRAN
Toos	STEPHENS/MCDERMID//CAMA/3/NEUZUCHT	Spring	CYMMIT
Bam	VEERY/NACUZARI-76//1-66-24	Spring	CYMMIT
Bayat	PUNJAB-76/CHENAB-70	Spring	CYMMIT
ArasGolsor	Landrace	Winter	KURDISTAN (IRAQ)
Arya ^T	GDOVZ469/3/JO_1//ND61.130/LDS	Spring	CYMMIT
Dez	KAUZ, MEX*2/OPATA-85//KAUZ, MEX	Spring	CYMMIT
Sepahan	AZADI/5/L2453/1347/4/KAL//BB/KAL/3/Y50E/3*KAL	Spring	
Khazar1	MEXICAN-TYPE/ROSHAN; 200-H/VILUFEN//ROSHAN	Facultative	CYMMIT
Omid	ERYTHROSPERMUM-59/TERTSIYA/4*TERTSIYA	Winter	
Rashagol	Landrace	Winter	KURDISTAN (IRAQ)
Darya	CHIL/SHA4	Spring	IRAN
Shoale	Landrace	Spring	IRAQ
Hamoon	Falat/Roshan	Spring	CYMMIT
Atrak	JUPATECO-73/(SIB)BLUEJAY//URES-81	Spring	CYMMIT
Navid	HYSLOP/SIETE-CERROS-66; MINHARDI/ODIN,SWE	Facultative	CYMMIT
Gaspard	ARMINDA/FD-71036		FRANCE
Zarin	NAINARI-60/HEINES-VII//BUCKBUCK/3/F-59-71/GOSHAWK	Facultative	CYMMIT
R2ERW-87	-	-	-
C-80-6	Bow"s"/Crow"s"/Kie"s"/Vee"s"	-	ICARDA
Mahdavi	TANORI-F-71/PICHON/5/MT-48/3/WILLET-ENANO*3//NARINO-59/TOTA-63/4/MUSALA	Spring	CYMMIT
Yavaros ^T	JORI-69(SIB)/(SIB)ANHINGA//FLAMINGO,MEX	Spring	CYMMIT
Kave	FORUNA/PALOMA	Spring	CYMMIT
Karkheh ^T	SHWA/MALD//AAZ/3/SRN	Spring	CYMMIT
Alborz	PITIC-62(I-276402)/BOGARNAYA-56//DNEPROVSKAYA-521/3/DAKOTA	Spring	CYMMIT
Chenab	C-271/WILLET-DWARF//SONORA-64	Spring	PAKISTAN
Golspi	Landrace	Winter	KURDISTAN (IRAQ)
Pishgam	BARAKAT/ZHONG87-90	Facultative	IRAN
DN-11	Azd/HD2172//Kayson/Glenson/3/1-70-28/Ning8201		

SorkhTokhm	Landrace		Spring	IRAN
Kavir	SHORTIM/3/KALYANSONA//V-534/JIT-716		Spring	CYMMIT
Tabas	Landrace		Spring	IRAN
Gaskogen	TJB-900-8/MARENGO		Winter	FRANCE
Akbari	1-63-31/3/12300/TOB//CNO67/SX		Spring	IRAN
Karaj3	DRUCHAMP/(MX)MEXICO-120/3/SONORA-64		Spring	IRAN
Moghan1	LERMA-ROJO//NORIN-10/BREVOR/4/YAKTANA-54//NORIN-10/BREVOR/3/3*ANDES		Spring	CYMMIT
Karaj1	MEXICAN-TYPE/ROSHAN; 200-H/VILUFEN//ROSHAN		Facultative	CYMMIT
Rasool	KAVKAZ=VEERY/(SIB)BUHO//KALYANSONA/BLUEBIRD		Spring	CYMMIT
Adl	TK/SHAHPASSAND		Spring	CYMMIT
Darab2	MAYA-74/NACAZARI-F-76		Spring	CYMMIT
Pishtaz	ALVAND//ALDAN/IAS-58		Spring	CYMMIT
Shirodi	ND/VG9144//KAL/BB/3/YACO/4/VEE#5		Spring	CYMMIT
BCRoshan	Rsh2-10120		-	ISFAHAN (IRAN)
LineA	-			AMERICA
Veenak	Veery/Nacozari or Veery#5/NacozariF76		Spring	CYMMIT
Hirmand	BAYAT/5/JARAL-F-66//CENTRIFEN/CIANO-67/3/SARIC-70/4/(SIB)JUPATECO-73		Spring	CYMMIT
Shahpasand	Landrace		Winter	SAVEH (IRAN)
Azar2	KAVKAZ/TANORI-71/3/MAYA-74(SIB)//BLUEBIRD/INIA-66/4/SEFID		Winter	CYMMIT
R1ERW-87	-			
Falat	KAVKAZ/(SIB)BUHO//KALYANSONA/BLUEBIRD		Spring	CYMMIT
Sabalan	908//FRONTANA/A-12/3/1.32.4382		Facultative	CYMMIT
Shahryar	KAVKAZ/TANORI-71/3/MAYA-74(SIB)//BLUEBIRD/INIA/4/KARAJ-2/5/ANZA/3/PITIC-62/NDR//HYSLOP		Winter	CYMMIT
Niknezhad	134F – 71/s" "/crow F-134-71/(SIB)CROW		Spring	CYMMIT
Arta	HD2206/Hork//Bul/6/CMH80A.253/2/M2A/CML//Ald*4/5/BH1146/H56.71//BH1146/3/CMH78.390/4/Seri 82/7/Hel/3*Cno79/7/2*Ser 82		Spring	IRAN
R3ERW-87	-			
M-79-7	ICW84-0008-013AP-300L-3AP-300L-0AP			ICARDA
C-81-4	Fin/Acc//Ana/3pew "s"/4F12:71/Coc//Cno79Shahi/Kvz/5/Shahi/4/Kal/Bb/ CJ "s"/ Hork "s"CrossedShahi			ICARDA
Karaj2	FALAT//THATCHER/MARTIN/3/OMID		Winter	
Moghan3	Luan/3/V763.23/V879.C8//Pvn/4/Picus/5/Opata		Spring	IRAN

T: Tetraploid Durum Wheat

Specification of grain Fe and Zn content

The grain samples (approximately 0.5 gr) were digested in a mixture of Chloridric acid (HCL) and Perchloridric acid (HCL04), according to Singh et al. (1999). The digested samples were analyzed for iron (Fe) and zinc (Zn) content (expressed as mg Kg⁻¹ dry weight) using flame atomic absorption spectroscopy (SpectrAA220-Varian Ltd., Mulgrave, Australia) in three replications. Appropriate quality control was carried out for each set of measurements.

Analysis of variance (ANOVA) and combined analysis of variance was conducted for comparison of the grain Fe and Zn content of the wheat genotypes each year and over the three years using UNIANOVA syntax SPSS 18.0, IBM. The bivariate correlation between the grain Fe and Zn contents of the wheat genotypes was analyzed through the Pearson coefficient (SPSS 18.0). Broad-sense heritability (H²) was estimated for grain Fe and Zn content on the basis of a combined three-year analysis using the following formula:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r} + \frac{\sigma_{\epsilon}^2}{nr}}$$

where σ_g^2 , σ_{gy}^2 , σ_e^2 , Y, and R represent the variances of the genotypes, genotype-year interaction, error, number of years, and number of replications per trial, respectively.

DNA isolation and PCR amplification

The collected leaves (3–4 young seedlings) were resorted to a -40°C freezer before DNA extraction. The DNA was isolated from the leaves using the CTAB method (Saghai-Marouf et al. 1984). The quality and quantity of the extracted DNA were tested using an 0.8% agarose gel and a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively. The isolated DNA was diluted to 50 ng/ μL for marker analysis. Twenty SCoT markers (Collard and Mackill 2009; Luo et al. 2010), twenty ITAP markers (Xiong et al. 2013), and twenty SSR markers were utilized for PCR amplification (Supplementary Table S2, S3). The SSR markers were selected and synthesized based on information from previous studies on wheat and the GrainGenes database (<https://wheat.pw.usda.gov/GG2>). The PCR amplification reaction was prepared in a final volume of 10 μL , consisting of 4.7 μL ddH₂O, 2 μL genomic DNA, 2.1 μL PCR master kit, and 1.2 μL of either primer (SCoT or ITAP) or 0.6 μL of the SSR forward and reverse primers. The following program was used for PCR amplification: 94 for 3 min followed by 35 cycles of 94 for 1 min, annealing temperature (48–68) for 1 min and 72 for 2 min, and final extension at 72 for 5 min. The amplified SCoT and ITAP products were separated using 1.3% agarose, and the SSR alleles were separated on denaturing polyacrylamide (6% acrylamide) gel and resolved through silver staining. The SSR alleles were scored as codominant markers based on band size. The binary codes 1 and 0 were also used for scoring both SCoT and ITAP amplicons.

Genetic data analysis

The Bayesian method (Markov Chain Monte Carlo, MCMC) was used to determine the possible subpopulations in the current wheat genotypes using ITAP, SCoT, and SSR genetic data. The STRUCTURE software version 2.3.4 (Pritchard et al. 2000) was utilized for specification of subpopulations. For the first run of STRUCTURE, the length of the burn-in period and the number of MCMC replications after burn-in were set to 100000 and 100000 (Eltaher et al. 2018), respectively for specification of the range of K subpopulations based on the minimum value of LnP(D) . Next, three iterations were defined with a burn-in period of length 100000, equal to the number of MCMC replications. The best value of K was determined based on Evanno et al. (2005) using the STRUCTURE Harvester website (Earl and von Holdt 2012). Accordingly, the most likely subpopulations were specified. Principal coordinate analysis (PCoA) was also conducted based on Nei's genetic distances and hierarchical analysis of molecular variance (AMOVA) using the GenALEX software version 6.501 (Peakall and Smouse 2012). The numbers of subpopulations defined by STRUCTURE were used for AMOVA. After the division of the seventy wheat genotypes into subpopulations, the genetic diversity statistics were calculated using GenALEX 6.501. These included expected heterozygosity (H_e), Shannon's information index (I), percentage of polymorphism (%P), number of different alleles (N_a), and number of effective alleles (N_e) (Peakall and Smouse 2012). Based on the SSR primers, the inbreeding coefficient (F_{is}), fixation index (F_{st}), and Hardy-Weinberg equilibrium were also estimated using GenALEX, and the polymorphism information content (PIC, Botstein et al. 1980) was estimated using the Cervus software version 3.0.7. The probability of identity (PI) was computed based on $PI = 2(\sum p_i^2)^2 - \sum p_i^4$, where p_i is the i^{th} SSR allele (Yang et al. 2016). The effective multiple ratios (EMR) and marker index (MI) characters were computed for comparison of the SCoT and ITAP marker systems. For specification of the informative markers for grain Fe and Zn content, the stepwise regression analysis and Pearson correlation test were performed using SPSS 18.0, IBM, and the General Linear Model (GLM) analysis was made using TASSEL 5.2.66 (Bradbury et al. 2007).

Results

Grain Fe and Zn Content

We evaluated Fe and Zn microelements in the grains of each wheat genotype on dry weight bases across the three consecutive years. The grain Fe and Zn content was distributed normally every year, indicating the polygenic nature of Fe and Zn, controlling genes. In the first year (2017), the grain Fe content averaged $59.65 \pm 9.16 \text{ mg Kg}^{-1}$, varying between 40.84 (Karkheh) and 85.24 (Hirmand). The grain Zn content was $54.61 \pm 6.76 \text{ mg Kg}^{-1}$ on average, ranging from 38.74 (Moghan3) to 73.24 (Gaspard). In the second year (2018), grain Fe content varied from 30.66 (Chamran) to 61.88 (Gaspard), with a mean of $44.75 \pm 5.82 \text{ mg Kg}^{-1}$. Grain Zn content was variable between 32.43 (Aras GolSoor) and 67.59 (Gaspard), with a mean of $45.95 \pm 8.68 \text{ mg Kg}^{-1}$. In the third year (2019), the maximum, minimum, and average grain Fe content was 90.26 (Shahpasand), 28.2 (Khazar1), and $54.73 \pm 14.29 \text{ mg Kg}^{-1}$, respectively. The grain Zn content averaged $47.92 \pm 8.42 \text{ mg Kg}^{-1}$, ranging from 33.67 (Zarin) to 70.76 (Shahpasand). Analysis of variance (based on the RCBD model) was performed separately for each year. ANOVA indicated that the wheat genotypes were significantly different every year in terms of grain Fe and Zn content ($p < 0.001$). A comparison of means was conducted using the Bonferroni method, and the results are presented in Fig. 1.

The error variances for every year were homogenate, according to Bartlett's test of equality of variances. Therefore, a combined analysis of variance was made to verify the interaction between grain Fe and Zn content and their environment. The effects of the years and of the interaction between the years and genotypes were highly significant for grain Fe and Zn content ($p < 0.001$, Table 2). Regardless of the significant year-genotype interaction, seven genotypes (including Hirmand, Gaspard, Arya, Shahpasand, CrossShahi, Marvdasht, and Kavir) were ranked highly with respect to grain Fe content in all the three years. Similarly, eleven genotypes (including Gaspard, Sabalan, Shahpasand, Karaj1, Karkheh, Hirmand, Arvand, Rasool, Shoeleh, Azar2, and Yavaros) were ranked highly for grain Zn content every year. High broad-sense heritability was estimated for grain Fe (89.29%, 76.51%, and 95.11%) and Zn (74.19%, 88.73%, and 84.48%) content in different years. The σ_g^2/σ_{gy}^2 ratio was calculated after the combined analysis of data, which was less than one for grain Fe and Zn content.

Table 2
Combined analysis of variance for grains Fe and Zn concentration

SOV	df	Mean of Squares	
		Fe	Zn
Year	2	10027.03	3093.95
Block (Year)	4	162.49	127.58
Genotype	69	405.31	245.03
Genotype × Year	138	188.12	113.78
Error	275	22.72	44.13
CV		9.19%	13.47%
H ²		53.59%	74.84%

The Pearson correlation test was conducted separately for each year between the grain Fe and Zn contents. The correlation of grain Fe and Zn content for the first year was not significant ($r = 0.230$, $p > 0.05$). In contrast, significant positive correlations were estimated between grain Fe and Zn contents in the second ($r = 0.477$, $p < 0.001$) and third ($r = 0.274$, $p < 0.05$) years.

Genetic Diversity

SSR Primers

Of the 20 SSR primer pairs assessed in the wheat genotypes, 15 produced unambiguous, polymorphic amplicons. The total number of generated alleles in the 70 wheat genotypes was 67, with the number of alleles per locus averaging 4.67 and ranging from 2 (edm16) to 9 (wmc617). The effective number (N_e) was 2.628 on average (variable from 1.116 to 6.011). Mean expected heterozygosity (H_e) was 0.539, with the variable ranging from 0.094 (barc48) to 0.833 (wmc617). Shannon's information index (I) ranged from 0.199 (barc48) to 1.931 (wmc617), with an average of 0.979. The values of PIC varied between 0.086 (barc48) and 0.831 (wmc617), with a mean value of 0.534. The greatest, lowest, and average values of haploid diversity (H) were 0.78, 0.07, and 0.536, respectively. The positive mean value of F_{is} (0.607) indicated the excess of homozygosity. The calculated average F_{st} value was 0.1, while F_{st} ranged from 0.005 (edm96) to 0.240 (gwm18). The barc48 (0.835) and wmc617 (0.04) loci exhibited the highest and lowest PI values, respectively. The frequencies of 34.82% of the alleles were lower than 0.1, and the accumulative PI value was 1.02×10^{-10} , indicating the likelihood of the same SSR profile for two randomly selected individuals (Table 3).

Table 3

Genetic diversity characteristics of SSR markers comprising number of observed alleles (N), number of different alleles (Na), number of effective alleles (Ne), polymorphism information content (PIC), haploid diversity (H), Shannon's information index (I), expected heterozygosity (He), probability of identity (PI), inbreeding coefficient (Fis), and fixation index (Fst)

Markers	N	Na	Ne	PIC	H	I	He	PI	Fis	Fst
barc29	4	3.67	2.11	0.48	0.51	0.87	0.46	0.28	0.82	0.20
barc67	4	3.33	2.02	0.45	0.55	0.83	0.48	0.23	1.00	0.14
barc83	3	3.00	2.38	0.56	0.62	0.95	0.58	0.21	0.73	0.09
barc98	4	3.67	2.85	0.62	0.67	1.13	0.65	0.16	0.92	0.04
barc48	4	2.00	1.12	0.09	0.09	0.20	0.09	0.84	1.00	0.04
gwm18	3	2.67	1.91	0.44	0.52	0.70	0.42	0.31	1.00	0.24
gwm149	4	4.00	2.65	0.65	0.63	1.09	0.62	0.14	0.22	0.14
gwm11	4	2.67	2.24	0.55	0.61	0.83	0.52	0.22	1.00	0.15
gwm46	8	6.33	4.02	0.76	0.78	1.57	0.75	0.07	0.70	0.05
gwm95	5	4.00	2.82	0.69	0.72	1.15	0.63	0.12	0.86	0.15
gwm160	4	4.00	3.18	0.64	0.56	1.23	0.68	0.15	-0.13	0.03
gwm219	5	3.33	2.57	0.62	0.67	0.99	0.60	0.16	0.87	0.11
wmc617	9	8.67	6.01	0.83	0.77	1.93	0.83	0.04	-0.03	0.02
edm16	2	2.00	1.39	0.23	0.27	0.40	0.25	0.57	1.00	0.10
edm96	4	2.67	2.15	0.42	0.07	0.80	0.53	0.33	-0.85	0.01
Mean	4.47	3.73	2.63	0.53	0.54	0.98	0.54	0.26	0.61	0.10
Total	67.00	56.00	39.42	-	-	-	-	1.02×10 ⁻¹⁰	-	-
ns, **, and *** means nonsignificant and significant at 0.01 and 0.001 deviations from Hardy-Weinberg Equilibrium										

SCoT Primers

Totally, 351 amplicons were produced using SCoT markers, of which 329 were polymorphic. The number of amplicons per primer averaged 17.55, and ranged from 12 (SCoT35) to 21 (SCoT29/36, SCoT20). The effective number (Ne) varied between 1.378 (SCoT35/2) and 1.81 (SCoT33), averaging 1.619. The highest and lowest values of expected heterozygosity (He) were 0.442 (SCoT33) and 0.237 (SCoT35/2), with a mean value of 0.350. Average Shannon's information index was 0.515, and the index ranged from 0.355 for SCoT14 to 0.632 for SCoT33. The maximum values of MI and EMR were obtained for combined SCoT29/36 (19.74, 5.98), and the minimum values for SCoT14 (9.4, 1.786) (Table 4).

Table 4

Genetic diversity characters of SCoT and ITAP markers comprising Number of total amplicons (N), number of polymorphic amplicons (NP), percentage of polymorphism (P%), amplicon frequency (AF), number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), expected heterozygosity (He), effective multiple ratio (EMR), and marker index (MI)

Markers	N	NP	P%	AF	Na	Ne	I	He	EMR	MI
SCoT										
SCoT14	15.00	10.00	66.67	0.65	1.67	1.40	0.36	0.24	9.40	1.79
SCoT35	12.00	12.00	100.00	0.55	2.00	1.62	0.54	0.36	11.28	3.28
SCoT1	19.00	18.00	94.74	0.58	1.95	1.67	0.52	0.36	16.92	4.69
SCoT36	18.00	18.00	100.00	0.53	2.00	1.71	0.57	0.39	16.92	5.14
SCoT29	16.00	16.00	100.00	0.55	2.00	1.71	0.58	0.40	15.04	4.72
SCoT27	16.00	16.00	100.00	0.45	2.00	1.59	0.53	0.35	15.04	4.23
SCoT33	17.00	17.00	100.00	0.59	2.00	1.81	0.63	0.44	15.98	5.47
SCoT70	20.00	20.00	100.00	0.45	2.00	1.61	0.51	0.34	18.80	5.09
SCoT12	19.00	19.00	100.00	0.43	2.00	1.58	0.53	0.35	17.86	5.04
SCoT22	17.00	17.00	100.00	0.53	2.00	1.66	0.52	0.36	15.98	4.47
SCoT20	21.00	21.00	100.00	0.39	2.00	1.54	0.48	0.32	19.74	5.01
SCoT31	17.00	17.00	100.00	0.64	2.00	1.77	0.61	0.43	15.98	5.29
SCoT2	20.00	15.00	75.00	0.61	1.75	1.48	0.40	0.27	14.10	3.00
SCoT10	13.00	12.00	92.31	0.46	1.92	1.49	0.47	0.31	11.28	2.82
SCoT34	18.00	13.00	72.22	0.73	1.72	1.47	0.39	0.27	12.22	2.57
SCoT29/36	21.00	21.00	100.00	0.58	2.00	1.70	0.56	0.39	19.74	5.98
SCoT36/1	20.00	20.00	100.00	0.62	2.00	1.80	0.61	0.42	18.80	6.11
SCoT29/1	18.00	18.00	100.00	0.61	2.00	1.69	0.57	0.39	16.92	5.19
SCoT3122	16.00	15.00	93.75	0.69	1.94	1.73	0.56	0.39	14.10	4.29
SCoT35/2	18.00	14.00	77.78	0.56	1.78	1.38	0.37	0.24	13.16	2.57
Mean	17.55	16.45	93.62	0.56	1.94	1.62	0.52	0.35	15.46	4.34
Total	351.00	329.00	1872.46	11.20	38.73	32.38	10.30	7.01	309.26	86.75
ITAP										
ITAP4/EM7	18.00	16.00	88.89	0.45	1.89	1.43	0.40	0.26	14.24	3.00
ITAP1/EM9	14.00	11.00	78.57	0.60	1.79	1.48	0.42	0.28	9.79	2.21
ITAP2/EM4	17.00	12.00	70.59	0.65	1.71	1.44	0.37	0.25	10.68	2.14
ITAP3/EM11	24.00	21.00	87.50	0.58	1.88	1.55	0.46	0.31	18.69	4.62
ITAP4/EM9	16.00	16.00	100.00	0.44	2.00	1.43	0.43	0.27	14.24	3.19
ITAP1/EM4	17.00	16.00	94.12	0.55	1.94	1.62	0.51	0.35	14.24	3.87
ITAP4/EM4	16.00	15.00	93.75	0.51	1.94	1.57	0.48	0.33	13.35	3.44
ITAP4/EM11	17.00	13.00	76.47	0.65	1.77	1.45	0.39	0.26	11.57	2.38
ITAP3/EM7	18.00	15.00	83.33	0.63	1.83	1.46	0.42	0.28	13.35	3.00
ITAP4/EM8	17.00	14.00	82.35	0.68	1.82	1.55	0.46	0.31	12.46	3.04
ITAP5/EM9	17.00	17.00	100.00	0.46	2.00	1.56	0.48	0.32	15.13	3.86
ITAP3/EM 9	12.00	12.00	100.00	0.35	2.00	1.47	0.44	0.29	10.68	2.49
ITAP3/EM10	15.00	13.00	86.67	0.52	1.87	1.53	0.44	0.30	11.57	2.74
ITAP5/EM5	14.00	13.00	92.86	0.56	1.93	1.61	0.52	0.35	11.57	3.25
ITAP5/EM4	17.00	14.00	82.35	0.68	1.82	1.62	0.50	0.35	12.46	3.36
ITAP4/EM10	18.00	18.00	100.00	0.53	2.00	1.66	0.54	0.37	16.02	4.63

Markers	N	NP	P%	AF	Na	Ne	I	He	EMR	MI
ITAP3/EM3	18.00	18.00	100.00	0.47	2.00	1.59	0.53	0.35	16.02	4.50
ITAP1/EM3	14.00	14.00	100.00	0.58	2.00	1.74	0.61	0.42	12.46	4.10
ITAP1/EM8	15.00	13.00	86.67	0.63	1.87	1.62	0.51	0.35	11.57	3.19
ITAP5/EM6	12.00	11.00	91.67	0.34	1.92	1.33	0.33	0.21	9.79	1.66
Mean	16.30	14.60	89.79	0.54	1.90	1.54	0.46	0.31	12.99	3.23
Total	326.00	292.00	1795.78	10.86	37.96	30.70	9.24	6.20	259.88	64.70

ITAP Primers

The ITAP markers generated a total of 326 amplicons, of which 292 were polymorphic. The number of amplicons per primer averaged 16.3, variable between 12 (ITAP5/EM6, ITAP3/EM9) and 24 (ITAP3/EM11). The effective number (N_e) varied from 1.332 (ITAP5/EM6) to 1.744 (ITAP1/EM3), with a mean of 1.535. The maximum and minimum values of expected heterozygosity (H_e) were 0.419 (ITAP1/EM3) and 0.206 (ITAP5/EM6), and the average value was 0.310. Shannon's information index (I) was 0.462 on average, ranging from 0.333 for ITAP5/EM6 to 0.607 for ITAP1/EM3. The average, highest, and lowest values of MI were 3.235, 4.63 (ITAP4/EM10), and 1.664 (ITAP5/EM6). The mean value of EMR was 12.994, and the values varied between 16.02 (ITAP4/EM10, ITAP3/EM3) and 9.79 (ITAP5/EM6, ITAP1/EM9) (Table 4).

Population Structure

SSR Data

The STRUCTURE software was used for specification of the population structures of the seventy wheat genotypes in Iran using SSR, ITAP, and SCoT data. Based on the SSR data, the peak was obtained at $K = 3$ after the number of clusters was plotted against ΔK (Evanno et al. 2005), indicating that the current wheat genotypes could be divided into three subpopulations (Fig. 2). The average ancestry values (Q values) of the individuals belonging to each genetic cluster varied from 0.297 to 0.362. The average distances (expected heterozygosity) between the individuals in the clusters were 0.5638, 0.4675, and 0.3422.

The maximum values of the parameters of genetic diversity ($I = 1.080$, $H_e = 0.594$) were estimated for subpopulation III, and the minimum values ($I = 0.883$, $H_e = 0.478$) were calculated for subpopulation I. The positive values of F_{is} in all the subpopulations indicated predominant homozygosity. Private alleles were found in the three subpopulations, ranging from 1 (subpopulation I) to 7 (subpopulation III), and allele frequency varied between 3.6% and 28.9%. Nei's genetic distance between the subpopulations was calculated, ranging from 0.193 (between subpopulations I and II) to 0.225 (between subpopulations I and III). The F_{st} value was calculated as an index for evaluation of total genetic diversity among the subpopulations. The maximum F_{st} value (0.084) was estimated between subpopulations I and III, while the minimum value was calculated between subpopulations II and III. The AMOVA indicated that most of the variation was there within the three subpopulations, 88%, whereas a significant variation of 12% could be observed among them ($p < 0.001$, Table 5). Most of the SSRs were not in Hardy-Weinberg equilibrium in any of the three subpopulations; the exceptions included *gwm149* in subpopulation I, *gwm60* in subpopulations I and II, and *wmc617* in all the subpopulations.

Table 5 – Molecular Analysis of Variance (AMOVA) based on SSR, SCoT, and ITAP data

Markers	Source	df	SS	MS	Est. Var.	%	PhiPT	P-Value
SSR								
	Among Populations	2	120.995	60.497	1.982	12%	0.118***	0.001
	Within Populations	67	992.663	14.816	14.816	88%		
	Total	69	1113.657		16.798	100%		
SCoT								
	Among Populations	3	517.324	172.441	6.795	11%	0.107***	0.001
	Within Populations	66	3748.933	56.802	56.802	89%		
	Total	69	4266.257		63.597	100%		
ITAP								
	Among Populations	5	1738.017	347.603	28.109	49%	0.493***	0.001
	Within Populations	64	1851.268	28.926	28.926	51%		
	Total	69	3589.286		57.035	100%		

Based on the principle PCoA analysis, the wheat genotypes were divided into three groups, approximately in accordance with the STRUCTURE analysis results (Fig. 3).

SCoT Data

The genotypes were assigned to four subpopulations ($K = 4$) based on the SCoT markers, Structure analysis, and ΔK calculation (Fig. 2). The mean Q value for each individual belonging to one of the four clusters was variable between 0.198 and 0.290. The average distances between the individuals in the four clusters were 0.4773, 0.522, 0.4715, and 0.4633, in that order. The wheat genotypes were partitioned into four subpopulations on the basis of the Nei's distance coefficient and PCoA analysis (Fig. 3). The subdivision groups were almost concordant with the results obtained by STRUCTURE. The parameters for the highest genetic diversity were calculated for subpopulation III ($N_e = 1.57$, $I = 0.481$, and $H_e = 0.326$), and the lowest variation was estimated for subpopulation I ($N_e = 1.444$, $I = 0.379$, and $H_e = 0.256$). Private amplicons were found in three of the four subpopulations, and the maximum number (5) was estimated for subpopulation III. Based on the AMOVA results, the highest percentage of variation was estimated within the subpopulations (89%), while the variation among the subpopulations (11%) was significant ($p < 0.001$, Table 5).

ITAP Data

Based on the ITAP data, the best value of K was calculated through the STRUCTURE software and the STRUCTURE Harvester website. The wheat genotypes were attributed to six subpopulations (Fig. 2). The average Q value of each individual in each subgroup varied from 0.538 to 0.998. The mean distances between the individuals in each subgroup were 0.3126, 0.3388, 0.3397, 0.4074, 0.1793, and 0.2929. Based on the PCoA analysis, the wheat genotypes were subdivided into five distinct groups closely consistent with the STRUCTURE clusters (Fig. 3). The statistics on maximum genetic diversity, including $N_e = 1.35$, $I = 0.30$, and $H_e = 0.20$, were measured in subgroup VI, and the minimum values ($N_e = 1.21$, $I = 0.174$, and $H_e = 0.12$) were obtained for subgroup III. Private amplicons were found in five out of the six subgroups, of which subgroup I involved the maximum number (6). Using AMOVA, The highest percentage of variation (51%) was estimated within the subgroups. Nonetheless, the percentage of variation between the subgroups (49%) was significant ($p < 0.001$, Table 5).

Informative Markers for the Grain Fe and Zn Content

The relevant informative or eventual SSR, SCoT, and ITAP for grain Fe and Zn content in the three years were examined using GLM, stepwise regression analysis, and Pearson's correlation test, and the results are presented in Tables 6–8. Some SSR amplicons pertaining to the *barc48* (4DL, 6BS), *gwm219* (6BL), *gwm160* (4A), and *wmc617* (4A, 4B, 4D) primers were associated with maximization and minimization of grain Fe and Zn content in the different years (Table 6). This possibly demonstrates the importance of their chromosome positions in the control of grain Fe and Zn contents. Approximately specific SCoT primers, including SCoT1, SCoT12, SCoT22, SCoT33, and the SCoT29/1 combination, were associated with higher and lower values of grain Fe and Zn contents in the three years (Table 7). Commonly, ITAP amplicons comprising ITAP1-EM4, ITAP3-EM10, ITAP1-EM9, ITAP4-EM10, ITAP1-EM9, ITAP3-EM11, and ITAP1-EM3 were correlated with lower and higher grain Fe and Zn contents in the different years, as presented in Table 8.

Table 6
 Informative SSR amplicons Based on GLM, stepwise regression, and Pearson correlation tests

Informative SSR Markers	Allele Size (bp)	GLM	Pearson Correlation	Chromosome Location
First Year Fe				
barc48	165	F = 5.04* R ² = 0.07	0.254*	4DL, 6BS
Second Year Fe				
barc98	360	F = 7.26** R ² = 0.094	-0.275*	4DS
barc83	250	F = 5.75* R ² = 0.079	-0.299*	1AL, 1BL, 7BL
wmc617	285	F = 5.10* R ² = 0.065	0.258*	4A, 4BS, 4D
Third Year Fe				
gwm160	300	F = 8.22** R ² = 0.112	0.346**	4AL
gwm149	350	F = 5.86** R ² = 0.082	0.270*	4BL
gwm11	295	F = 6.11* R ² = 0.084	0.287*	1BS
First Year Zn				
gwm160	350	F = 8.22** R ² = 0.105	0.354**	4AL
gwm160	400	F = 20.56*** R ² = 0.224	0.492**	4AL
gwm149	365	F = 4.39 R ² = 0.059	-0.279*	4BL
gwm149	380	F = 4.57* R ² = 0.061	0.280*	4BL
barc83	250	F = 16.26*** R ² = 0.187	-0.394**	1AL, 1BL, 7BL
gwm219	350	F = 8.52** R ² = 0.11	0.354**	6BL
barc48	150	F = 10.00** R ² = 0.126	0.344**	4DL, 6BS
barc48	155	F = 7.46** R ² = 0.098	-0.323**	4DL, 6BS
Second Year Zn				
gwm149	350	F = 6.84** R ² = 0.087	0.336**	4BL
barc29	245	F = 4.29* R ² = 0.05	-0.330**	7AL

*** significant in 5% and 1% respectively

Informative SSR Markers	Allele Size (bp)	GLM	Pearson Correlation	Chromosome Location
barc48	150	F = 4.38* R ² = 0.04	0.274*	4DL, 6BS
Third Year Zn				
gwm160	400	F = 12.51*** R ² = 0.161	0.407**	4AL
wmc617	270	F = 5.54* R ² = 0.078	-0.287*	4A, 4BS, 4D
edm96	188	F = 4.80* R ² = 0.069	0.258*	3AS
edm96	190	F = 7.1** R ² = 0.099	-0.258*	3AS
*** significant in 5% and 1% respectively				

Table 7
 Informative SCoT amplicons based on GLM, stepwise regression, and Pearson correlation tests

Informative SCoT Markers	Allele Size (bp)	GLM	Pearson Correlation
First Year Fe			
SCoT1	400	F = 6.74* R ² = 0.094	0.306**
SCoT1	800	F = 10.19** R ² = 0.14	0.314**
SCoT29/1	700	F = 7.68** R ² = 0.106	-0.320***
SCoT22	400	F = 8.11** R ² = 0.111	0.334***
SCoT35/2	800	F = 5.70* R ² = 0.081	0.298*
SCoT35/2	1500	F = 11.32** R ² = 0.1484	0.396***
Second Year Fe			
SCoT29/1	1200	F = 4.64* R ² = 0.062	-0.232ns
Third Year Fe			
SCoT1	400	F = 4.54* R ² = 0.056	0.247*
SCoT29/1	700	F = 10.30** R ² = 0.12	-0.329**
SCoT33	300	F = 6.72* R ² = 0.081	-0.320**
SCoT12	250	F = 5.96* R ² = 0.0723	-0.342**
SCoT12	300	F = 4.93** R ² = 0.061	-0.332**
SCoT12	450	F = 5.32* R ² = 0.065	-0.328**
SCoT22	400	F = 8.25* R ² = 0.097	0.392**
SCoT31/22	1600	F = 6.71** R ² = 0.081	-0.321**
First Year Zn			
SCoT33	1200	F = 5.51* R ² = 0.064	-0.335**
SCoT12	250	F = 5.17* R ² = 0.060	-0.331**
*** significant in 5% and 1% respectively			

Informative SCoT Markers	Allele Size (bp)	GLM	Pearson Correlation
SCoT22	550	F = 7.08** R ² = 0.080	-0.370**
SCoT35/2	700	F = 9.03** R ² = 0.10	0.315**
Second Year Zn			
SCoT1	1100	F = 12.82** R ² = 0.15	0.307**
SCoT1	1300	F = 10.30** R ² = 0.12	0.317**
SCoT1	1400	F = 10.30** R ² = 0.12	0.317**
SCoT12	1500	F = 10.48** R ² = 0.12	-0.392**
SCoT22	550	F = 6.11* R ² = 0.077	-0.338**
Third Year Zn			
SCoT12	250	F = 6.44* R ² = 0.082	-0.303*
SCoT22	400	F = 8.74** R ² = 0.1078	0.360**
SCoT22	550	F = 8.83** R ² = 0.1088	-0.330**
SCoT20	600	F = 4.85* R ² = 0.063	-0.332**
SCoT20	900	F = 5.17* R ² = 0.072	-0.364**
SCoT31	1100	F = 9.93** R ² = 0.12	0.321**
SCoT35/2	800	F = 5.07* R ² = 0.067	0.260*
**, * significant in 5% and 1% respectively			

Table 8
 Informative ITAP amplicons based on GLM, stepwise regression, and Pearson correlation tests

Informative ITAP Markers	Allele Size (bp)	GLM	Pearson Correlation
First Year Fe			
ITAP3-EM7	500	F = 9.85** R ² = 0.13	-0.293*
ITAP3-EM10	1700	F = 7.31** R ² = 0.099	-0.284*
ITAP3-EM10	1900	F = 12.30** R ² = 0.16	-0.334**
ITAP1-EM3	1100	F = 4.76* R ² = 0.067	0.309**
ITAP1-EM3	1200	F = 5.22* R ² = 0.073	0.313**
Second Year Fe			
ITAP1-EM9	200	F = 10.80** R ² = 0.13	0.322**
ITAP2-EM4	350	F = 4.64* R ² = 0.059	0.365**
ITAP2-EM4	600	F = 5.21* R ² = 0.066	-0.330**
ITAP5-EM5	400	F = 7.18* R ² = 0.088	0.307**
ITAP1-EM8	500	F = 11.061** R ² = 0.13	-0.455***
Third Year Fe			
ITAP1-EM4	1300	F = 13.55*** R ² = 0.17	-0.431***
ITAP1-EM4	1400	F = 11.12** R ² = 0.14	-0.374***
ITAP1-EM4	1500	F = 7.70** R ² = 0.10	-0.355**
ITAP5-EM9	1500	F = 11.061** R ² = 0.13	-0.314**
First Year Zn			
ITAP4-EM7	1500	F = 4.82* R ² = 0.067	0.306**
ITAP4-EM10	400	F = 9.71** R ² = 0.13	-0.263*
ITAP4-EM10	1100	F = 8.81** R ² = 0.11	0.269*
*** significant in 5% and 1% respectively			

Informative ITAP Markers	Allele Size (bp)	GLM	Pearson Correlation
Second Year Zn			
ITAP1-EM9	200	F = 4.89* R ² = 0.07	0.287*
ITAP1-EM9	1700	F = 6.01* R ² = 0.084	-0.322**
ITAP3-EM10	1700	F = 9.59** R ² = 0.13	-0.319**
ITAP4-EM10	400	F = 6.00* R ² = 0.084	-0.227*
ITAP5-EM6	1500	F = 7.22** R ² = 0.10	-0.346**
Third Year Zn			
ITAP4-EM10	1100	F = 7.37** R ² = 0.104	0.281*
*,** significant in 5% and 1% respectively			

Discussion

In addition to the fact that micronutrients are essential in human nutrition, and guarantee human health, they significantly affect the plant itself. Fe and Zn are involved in enzymatic functions, photosynthesis, and the metabolic and physiological processes that the plant undergoes (Peleg et al. 2009). For efficient enhancement of grain Fe and Zn content in a breeding program, therefore, it is vital to perceive microelement variation and find linked molecular markers. The variation in the grain Fe and Zn content of seventy prevalent wheat genotypes in Iran was assessed for three years. The results indicated considerable variation among the genotypes for grain Fe and Zn in each separate year. Several previous studies have reported significant variation in grain mineral content, including Fe and Zn, in cultivated and wild wheat relative genotypes (Cakmak et al. 2004; Velu et al. 2011, 2017; Srinivasa et al. 2014; Amiri et al. 2015; Gorafi et al. 2016; Pandey et al. 2016). The grain Fe and Zn contents varied in our study between 2.09 and 1.89 times, respectively, in the first year, 1.69 and 2.08 times, respectively, in the second year, and 3.2 and 2.10 times in the third. Similar ranges with two- or three-fold variation for grain Fe and Zn content have been reported in several previous studies on wheat populations (Velu et al. 2011; Amiri et al. 2015; Guttieri et al. 2015; Kenzhebayeva et al. 2019). To enhance the health influence and bioavailability of Fe and Zn via wheat grains, the contents have to be increased by up to 60.0 and 40.0 mg Kg⁻¹ (Cakmak 2008; Pandey et al. 2016). A number of our genotypes met these conditions. These genotypes could be used directly or as donors in breeding programs.

The different ranges and means and standard deviations of grain Fe and Zn contents each year are due to the environmental variation, indicating the impact of environmental factors such as agricultural practices and weather conditions. Moreover, genetic and environmental variation account for the different reported ranges of grain Fe and Zn content in various studies (Cakmak et al. 2004; Amiri et al. 2015; Pandey et al. 2016). A significant interaction between genotype and year revealed the important role of the genes controlling grains Fe and Zn content, as previously reported in other research (Peterson et al. 1986; Velu et al. 2011; Srinivasa et al. 2014; Guttieri et al. 2015; Gorafi et al. 2016). Constant ranking across the three years was exhibited by seven genotypes for grain Fe content (Hirmand, Gaspard, Arya, Shahpasand, CrossShahi, Marvdasht, and Kavir) and eleven genotypes for grain Zn content (Gaspard, Sabalan, Shahpasand, Karaj1, Karkheh, Hirmand, Arvand, Rasool, Shoeleh, Azar2, and Yavaros). These genotypes deserve consideration, and could be used as donor sources for increasing grain Fe and Zn content. The $\sigma^2_g/\sigma^2_{gy} < 1$ ratio indicated that the content is affected by the selection of environment conditions; therefore, successful breeding for enhancement of wheat grain mineral contents relies on the selection environment (Guttieri et al. 2015). In Peterson et al. (1986), the ratio was less than one for Ca, Zn, and Mn and more than one for Fe. In Guttieri et al. (2015), however, it was estimated to be more than one for Fe and Zn content in wheat grains. The lower proportion of genetic variation (σ^2_g) than that of genetic-environment interaction could be attributed to the complex nature of the grain Fe and Zn content controlled by polygenes (Khokhar et al. 2018). The genetic diversity of grain Fe and Zn content provides valuable sources for enhancement of the microelement concentration of wheat grains via traditional or molecular breeding approaches (Amiri et al. 2015). In our study, a high broad-sense heritability was estimated for grain Fe and Zn content, which had been reported in previous studies on bread wheat, wheat relative, and other cereal genotypes (Badakhshan et al. 2013; Khodadadi et al. 2014; Srinivasa et al. 2014; Goudia and Hash 2015; Gorafi et al. 2016). The significant broad-sense heritability could be accounted for by the impact of genetics on grain Fe and Zn content in wheat genotypes and the possibility of selection of genotypes with high Fe and Zn content in wheat genotypes.

A moderately positive correlation was estimated between grain Fe and Zn contents, as previously reported in similar studies on wheat (Cakmak et al. 2004; Srinivasa et al. 2014; Amiri et al. 2015; Pandey et al. 2016). This positive relation could be attributed either to respective co-segregation and co-localization alleles and QTLs or to the pleiotropic effects of genes such as major QTL (*Gps-B1*) controlling grain Fe, Zn microelements, and protein content (Cakmak et al. 2004; Distelfeld et al. 2007). The *Gps-B1* locus (on chromosome 6B) derived from the wild emmer wheat (*Triticum dicoccoides*) affects the remobilization of

protein, Zn, and Fe from leaves to the grain, and is also involved in the senescence of earlier flag leaves (Distelfeld et al. 2007; Coco et al. 2019). The positive correlation between micronutrients serves to improve wheat cultivars with enhanced microelements and protein contents (Goudia and Hash 2015) concurrently. A common molecular mechanism for grain micronutrient uptake and metabolism or common transporters could explain the positive correlation between grain Fe and Zn contents (Phuke et al. 2017).

The development of DNA-based molecular markers has enabled efficient specification of genetic diversity, QTL mapping, efficient gene pyramiding, and precise marker-assisted selection of linked targeted QTLs (Goudia and Hash 2015). We used the three marker systems SSR, ITAP, and SCoT to find informative amplicons for grain Fe and Zn content. Each marker system was designed and amplified for specific regions of the genome, including sequences flanking hypervariable regions of microsatellites (SSR), sequences flanking the start codon (ATG) in plant genes (SCoT, Collard and Mackill 2009), and 3' widely distributed conserved intron-exon junction sequences (ITAP, Xiong et al. 2013). The origin of polymorphism detected by ITAP markers could be intron length polymorphism and point mutations of ITAP binding sites. Therefore, variable markers with different genome target sequences enabled us to detect informative markers from different parts of the genome. Informative amplicons could be sequenced and developed as sequence-characterized amplified region (SCAR) markers for reliable, precise investigation of grain Fe and Zn content in a wheat genetic background.

Various informative SSR loci were identified for grain Fe and Zn content on chromosomes 1A, 3A, 4A, 7A, 1B, 4B, 6B, 7B, and 4D (Table 6), explaining 5.04 to 8.22 percent of the Fe content and 4.29 to 20.56 percent for Zn. It seemed that the homeologous group 4 chromosomes and the 6B chromosome played important roles in the control of grain Fe and Zn content in our wheat germplasm because these loci were detected commonly for grain Fe and Zn content. Homoeology has been reported previously by (Peleg et al. 2009) for Fe, Zn, protein, and other mineral content in the tetraploid wheat population. Although macro elements such as phosphorous (P) and sulfur (S) were not addressed in the present study, high positive correlation has been reported previously by Peleg et al. (2009) to be there between P, S, Fe, and Zn. Peleg et al. (2009) identified QTLs on the homeologous group 4 chromosomes for P and S content in tetraploid germplasm, reinforcing the co-localization of ascribed QTLs for Fe, Zn, P, and S, on similar chromosomes. They concluded that the QTL homoeology might indicate synteny between different genomes. Particular SSR loci, including *barc48*, *wmc617*, and *gwm160*, were commonly correlated with grain Fe and Zn content, demonstrating the co-localization or pleiotropic effect of genes controlling Fe and Zn content. Dissimilar correlated markers with grain Fe content were detected in the three years, presumably because of significant genotype-environment interaction (Table 6). Identical results were reported by Gorafi et al. (2016) for Fe and Zn concentrations of wheat grains. Several genes were identified in the cereals and Arabidopsis involved in Fe and Zn uptake, translocation, and storage, including YSL (yellow stripe-like), ZIP (iron-regulated transporter-like protein), NRAMP (natural resistance-associated macrophage protein), FER (ferritin-like), and NAS (nicotianamine synthase), belonging to the Fe and Zn super-families (Mahendrakar et al. 2020). Zhou et al. (2020) identified seven candidate genes for grain Zn accumulations in wheat. The genes encoded the NAC transcription factor on the 3D chromosome, a V-type proton ATPase on chromosome 4A, a protein containing tetratricopeptide (TPR) repeats on the 1B chromosome, serine/threonine-protein kinase on the 5B chromosome, a CTP synthase on the 3B chromosome, the basic helix-loop-helix transcription factor (BHLH) on the 7A chromosome, and heavy metal transport/detoxification superfamily protein on the 5A chromosome. The identified genes involved nutrient remobilization from leaves to wheat grains, cell ion homeostasis, osmotic stress response, metal ion binding, regulation of voltage-dependent ion channels, and Zn concentration in grains, respectively.

Particular SSR loci correlated with grain Zn content, comprising *gwm160*, *gwm149*, and *barc48*, were identified in two consecutive years. The *gwm160* locus located on homeologous group 4 was noteworthy as an informative marker. It was detected as highly positive significantly correlated alleles with a high capability of specifying grain Fe and Zn content (8.22–20.56%, Table 6) in the different years. The impacts of additive and epistatic QTLs on grain Fe and Zn content reported in previous studies have been located mainly on the chromosomes 2A, 4A, 5A, 7A, 5B, 6B, 7B, 2D, 3D, 4D, and 5D for grain Fe content and 2A, 3A, 5A, 6A, 7A, 2B, 4B, 5B, 6B, 7B, 1D, 2D, 3D, 4D, 5D, and 7D for grain Zn content (Shi et al. 2008; Peleg et al. 2009; Roshanzamir et al. 2013; Pu et al. 2014; Gorafi et al. 2016). Cakmak et al. (2004) reported that genes on chromosomes 6B, 5B, and 6A played an important role in raising Fe and Zn concentration levels in wild wheat relative *Triticum dicoccoides* substitution lines. According to Xu et al. (2011), D and S genomes of wheat positively affect the accumulation of Fe and Zn in grains. Mapping the microelement QTLs provides the basis of marker-assisted selection (MAS) to enforce efficient selection in conventional plant breeding, a pyramiding of genes enhancing microelement contents, also a basis for QTL cloning and gene transformation through genetic engineering approaches (Goudia and Hash 2015; Zhou et al. 2020).

Several informative SCoT amplicons were detected for grain Fe and Zn content in the three years (Table 7). The SCoT amplicons SCoT1 (400, 1300, 1400bp) and SCoT22 (400bp) were positively correlated with grain Fe and Zn content. Combined SCoT29/1 (700bp) was negatively correlated with grain Fe, while SCoT22 (550bp) and SCoT12 (250, 150bp) were negatively correlated, and SCoT35/2 (700, 800bp) was positively correlated with grain Zn content in the three years (Table 7). The SCoT22 (400bp) amplicon was repeatedly detected in the three years for high Fe and Zn content; thus, it should be considered and sequenced in future studies. Multiple informative ITAP amplicons were identified for grain Fe and Zn content in the different years (Table 8). Most of the identified amplicons were negatively correlated with grain Fe and Zn contents. However, the ITAP1-Em9 (200bp) amplicon was positively correlated with Fe and Zn content in the second year. The ITAP4-Em10 (1100bp) amplicon was revealed as positively correlated with Zn content in two different years. Variable ITAP amplicons were demonstrated in the three consecutive years for Fe content. Common informative SCoT and ITAP amplicons for both Fe and Zn content reflected the positive correlation of these elements in wheat grains.

Genetic Diversity

In this study, genetic diversity was estimated based on several indices, including effective number (N_e), expected heterozygosity (H_e), Shannon's information index (I), haploid diversity (H), and PIC. For the SSR markers, the average values of N_e , H_e , I , H , and PIC were 2.63, 0.54, 0.98, 0.54, and 0.53, respectively, indicating adequate genetic diversity among the present wheat population. The PIC values were calculated as more than 0.5 for 9 out of the 15 SSR primers, demonstrating highly informative SSR primers. Highly informative markers could be used for reliable genotyping and estimation of the genetic diversity of populations (Eltaher et al. 2018). The estimated H_e and PIC values were consistent with those in previous studies on wheat (Manifesto et al. 2001; Naghavi et

al. 2009; Senturk Akfirat and Uncuoglu 2013; Trkulja et al. 2019). Null alleles were present for nearly all of the utilized SSR primers, as confirmed by the positive values of Fis. The positive average value of Fis (0.61) indicated a significant deviation from the Hardy-Wienberg equilibrium and presence of more homozygous genotypes, which was not a surprising result for the self-fertilized wheat population.

The average values of Ne, He, and I for the SCoT markers were 1.619, 0.350, and 0.515, respectively. On the other hand, the mean values for the ITAP markers were Ne = 1.535, He = 0.419, and I = 0.462. These results indicated that the SCoT markers detected more genetic variability in the wheat population than the ITAP markers. Besides, the discriminating power of the SCoT and ITAP markers was compared based on the EMR and MI values, demonstrating that the SCoT markers could potentially be more efficient in detection of genetic diversity. Previous research has reported that SCoT markers have effectively indicated genetic diversity in the wheat population (Nasrollahi et al. 2019; Khodaei et al. 2021), but there is yet no reported study on application of ITAP markers to wheat genotypes.

Population Structures and Relationships

Based on the SSR data, the wheat genotypes were divided into three subgroups, in accordance with the PCoA results. The genotypes with the largest numbers, the highest consistency with the pedigree information, were classified as subpopulation III. The lowest-number genotypes were classified as subgroup I. However, the number of common parents of the genotypes in this group was larger than that in subpopulation II. Moreover, two tetraploid durum wheat genotypes (Karkheh and Arya) and five landraces with similar origins (specifically, RashaGol, GolSepi, and AarasGolsoor from the Kurdistan region of Iraq), were classified altogether into group I. The maximum numbers of private alleles, 0.594 (He) and 1.1080 (I), were calculated for subpopulation III. Private alleles are indicators of private genetic diversity, and loci with different alleles could potentially be useful in breeding programs (Eltaher et al. 2018). The majority of wheat genotypes ranked as possessing high grain Fe and Zn contents were categorized into subgroups I and III, respectively.

Using the SCoT data, the wheat genotypes were divided into four admixture subgroups. The number and quality of genotypes in each subpopulation were in line with those in the PCoA results. Most wheat genotypes were classified into subpopulation IV, with the largest number of common parents. Unlike in the SSR subpopulations, none of the above-mentioned landraces or tetraploid genotypes was grouped. Instead, two tetraploids, including Yavaros and Karkheh, were categorized in the same group. The highest genetic diversity (He = 0.326, I = 0.481) and the largest number of private amplicons (5) were estimated for subpopulation III, indicating proprietary genetic diversity within the subpopulation.

Based on the ITAP data, the wheat genotypes were portioned into six subpopulations. Using PCoA analysis, the wheat population was divided into five subpopulations, closely consistent with the results of the structure analysis. The largest subpopulations were V and VI, comprising 15 wheat genotypes each. However, subpopulation II exhibited the maximum consistency with the pedigree information. As in the SCoT-based subpopulations, two tetraploid wheat genotypes, including Yavaros and Karkheh, were grouped together. The greatest genetic variation (He = 0.2, I = 0.30) was estimated for subpopulation VI. In contrast, subpopulation I involved the maximum number of private alleles (6), indicating unique genetic diversity, presumably due to the presence of wheat genotypes with various, uncommon parents.

Based on the three different marker data, the numbers of subpopulations were different, but the groupings of the genotypes were identical in some cases. The two landraces GolSepi and Sorkhtokhm and cultivars like Golestan and Chamran, Rasool and Shiroodi, and Falat and Karaj2 were paired together due to equal parents, similar geo-ecological conditions, and presumably similar traits as considered by breeders. The SSR markers were the most consistent with the pedigree information, followed by SCoT and ITAP, in that order. This finding was not unexpected because of the specificity of the SSR primers.

The results of AMOVA using the SSR, SCoT, and ITAP data indicated high variation within the subpopulations (88, 89, and 51 percent, respectively). Nevertheless, the divergence among the subpopulations (12, 11, and 49 percent, respectively) was also significant ($p < 0.001$). The low genetic variation among the subpopulations could be attributed to the gene flow due to the presence of common parents in the subpopulations. It could be assumed that different growth habits, ecological adaptation, breeding goals, and ploidy possibly determined the genetic diversity within the subpopulations. Consistent results have been reported in earlier studies on wheat populations. In accordance with Eltaher et al. (2018), the distinct structure observed in our wheat population could be accounted for broadly in terms of common parents, common breeding goals for selection of genotypes, or common geographic conditions. Most of the studied genotypes were obtained mainly from the International Maize and Wheat Improvement Center (CIMMYT) breeding programs.

Conclusion

Biofortification of cereals, as a primary food source in developing countries, is recommended to prevent severe malnutrition and health problems. Substantial genetic variability was estimated for grain Fe and Zn content in the wheat genotypes examined in the current study. Variation in grain Fe and Zn content predisposes improvements in cultivars in terms of nutrient uptake from the soil, bioavailability, and grain filling. By the same token, association of informative molecular markers with responsible genes provides an efficient marker-assisted selection of genotypes and the possibility of pyramiding major genes in the ideal genotypes. A number of informative SSR, SCoT, and ITAP amplicons were identified, positively correlated with high Fe and Zn content. They could potentially be used to improve grain Fe and Zn content in wheat breeding programs. The markers considered here have to be further assessed in larger wheat populations using the association mapping approach. Some of the amplicons correlated with grain Fe and Zn content were constant across at least two years and common for grain Fe and Zn, reinforcing the co-segregation of the alleles involving these microelements. Regardless of the significant impact of the year-genotype interaction, a few stable genotypes were ranked highly for Fe, and Zn content could potentially be used in hybridization programs as donor parents. High genetic diversity among the wheat genotypes was revealed by the SSR, SCoT, and ITAP markers.

Declarations

Funding: No funding was received for conducting this study.

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

Availability of data and material: The dataset generated or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable

Authors Contribution Statement

Hedieh Badakhshan involved in all aspects of the research, designed the research, analyzed data, and wrote the manuscript. Rozhin Nosratifar conducted experiments. All authors read and approved the final manuscript.

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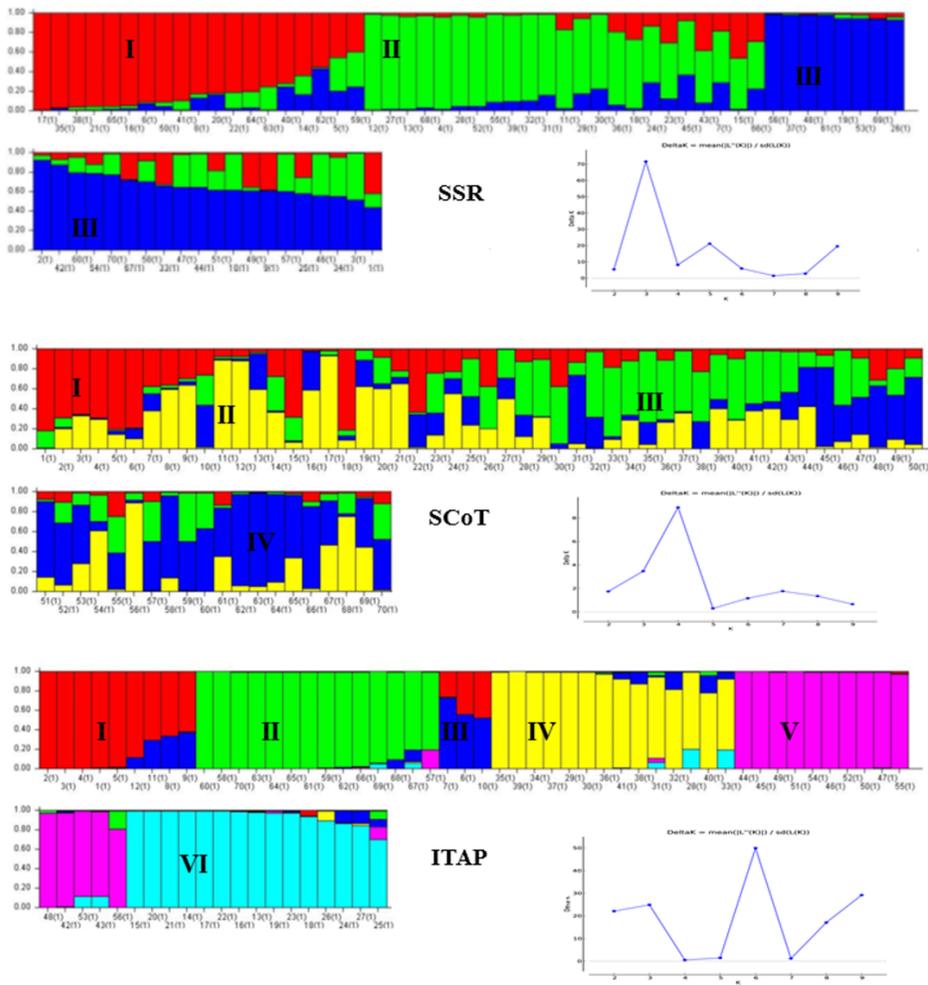


Figure 2
 Assignment of seventy wheat genotypes into three, four, and six subpopulations based on SSR, SCoT, and ITAP data using STRUCTURE software. Based on SSR, SCoT, and ITAP data, the ΔK values were estimated to speciate the number of groups in the wheat genotypes.

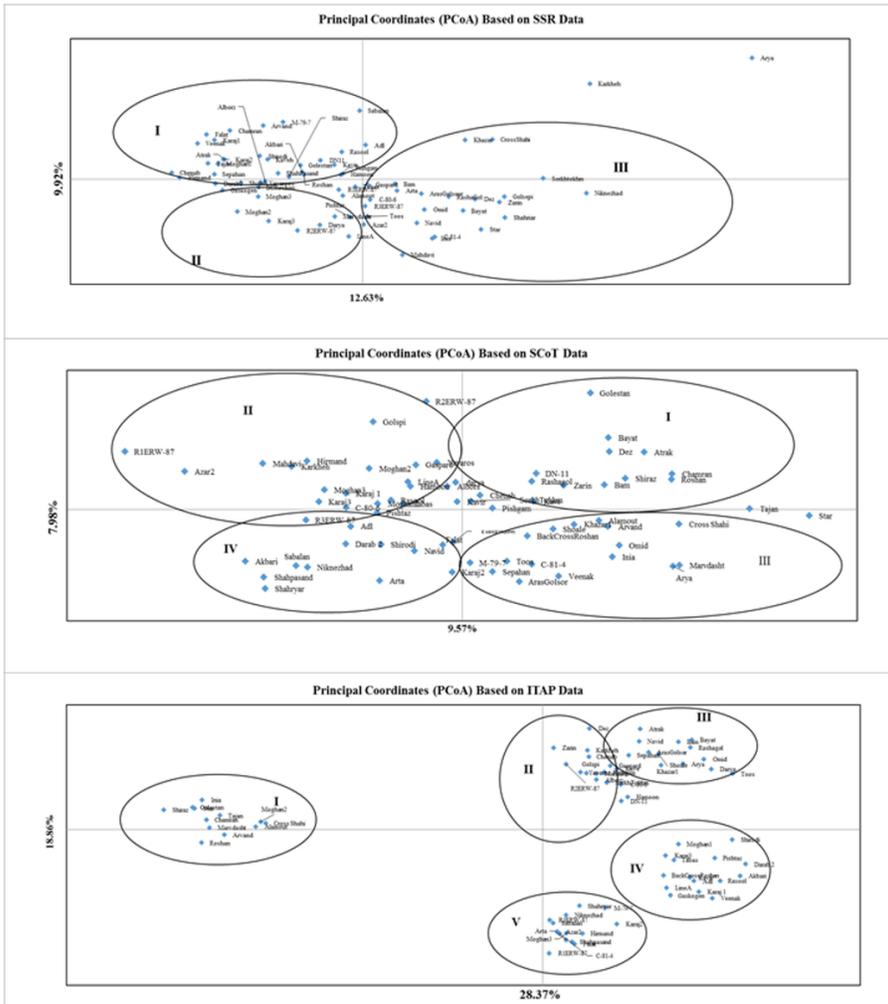


Figure 3

Division of seventy wheat genotypes based on Nei's distance coefficient and principal coordinate analysis (PCoA) using SSR, SCoT, and ITAP data.

Supplementary Files

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