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Assessment of genetic diversity and identification of SSR markers associated with grain iron content in Iranian prevalent wheat genotypes

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Abstract
Iron is one of the most important nutrients in the human diet. According to the high consumption of staple foods such as wheat, the deficiency of iron in these crops would lead to nutritional disorders and related complications. To identify microsatellite markers associated with wheat grain iron content,38Iranian prevalent wheat genotypes were assessed using 30 pairs of genomic and EST microsatellite markers. Based on field experiments, significant difference was observed among studied genotypes for grain iron content which ranged from 34-53 mg/Kg. in the molecular experiment, the range of alleles per SSR locus was 2-9 with a mean of 4.5 and the mean of polymorphism information content (PIC) was 0.55. The stepwise regression analysis has been used for estimating the relationship between microsatellite markers and grain iron content. The results indicated that Xwmc617 (4A, 4B, 4D), Xgwm160 (4A) and Xbarc146 (6D,6B,6A) were significantly correlated with wheat grain iron content. The results of this research can be used in further studies and marker assisted breeding of wheat to increase grain iron content.

Keywords: micronutrient, microsatellite marker, stepwise regression, wheat.

Introduction

Micronutrient deficiency and its consequences such as related malnutrition (hidden hunger) is one of the most important issues in the human health, especially in the poor and developing countries. Nearly half of the world's population is suffering from micronutrient deficiency. Unfortunately the main efforts in cereal breeding activities have been focused on increasing the yield without considering their nutritional quality (Bouis, 2003; Tiwari *et al*., 2009; Buis and Welch, 2010). Among the micronutrients, iron is important due to its key role in fundamental physiological processes, so its deficiency could cause nutritional disorders such as anemia, mental retardation, immune weakness, and even death (Raboy, 2009; Tiwari *et al*., 2009). Wheat is one of the most important cereal crops and plays a major role in the world's energy and protein supply. Although limited, increasing iron content in wheat, where

have no negative impact on yield performance, play a significant role in reducing malnutrition caused by the lack of this element (Raboy, 2009). Assessment of the genetic diversity and search for the relationship between the existing genotypes and desired traits and consequently identifying the trait-promised genotypes are the first most important steps in improving plants for various purposes, especially in the field of micronutrient content (Chacmak *et al*., 2004). Among different methods used for evaluating genetic diversity and relationship between the traits and existing diversity, molecular markers have particular importance. Among the molecular markers, microsatellite markers due to many advantages such as codominant effect, vast genome coverage, ease of detection, polymorphism and discrimination power are used in different studies such as, association between markers and traits as well as identification of genes and QTLs (Pirseyedi *et al*., 2006; Ahmadi and Fotokian, 2011; Tiwari *et al*., 2009; Peleg *et al*., 2009).

Identifying the correlation between markers and wheat traits, such as resistance to biotic and abiotic stresses, yield and nutritional quality, is one of the important fields of study in plant breeding (Qi *et al*., 2010). In several studies using SSR markers on different types of wheat genotypes ranging from wild diploid wheat to tetra and hexaploid genotypes including emmer, durum and bread wheat, several QTLs controlling concentration of micronutrients especially Fe, Zn and protein have been identified on wheat genome (Tiwari *et al*., 2009; Peleg *et al*., 2009).

The identification of markers associated with agronomic and morphological traits is another trend for research in molecular marker studies. In a study on evaluation of genetic diversity and identification of markers related to yield and plant height, 23 SSR loci have indicated a significant correlation with aforesaid traits (Wu *et al*., 2012). In Iran, however several studies have been performed on different plant attributes such as biotic and abiotic stresses using SSR markers but no study have been reported on wheat grain iron content yet.

The objective of the present study was to identify microsatellite markers that illustrate a significant correlation with wheat grain iron content using Iranian prevalent wheat genotypes and consequent identification of the chromosome regions controlling this trait according to the specified chromosomal location of SSR markers.

Material and methods

Plant materials and growth condition

A number of 38 genotypes of prevalent bread wheat cultivars prepared from the cereal part of "Karaj seed and plant improvement institute" Karaj, Iran were used. Field cultivation was performed in the Kurdistan University's research farm during 2010-2011 in a completely randomized block design with three replications of each cultivar and three rows per replication. When completely ripened, middle spikes from the middle row of each plot were randomly picked and kept separately in paper bags.

Grain iron content estimation

Grain iron content was measured using atomic absorption spectrometry Model (SpectrAA220) VARIAN Inc. which includes, the preparation of the meal, sample digestion for preparation of extract, preparation of standards and finally, sample readings. For the preparation of wheat meal, 15 to 20 grams of purified and isolated wheat was milled for 30 to 40 seconds. Extracts and iron standards were prepared according to Singh *et al*., (1999) with slight alterations.

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh leaves, based on Saghai-Maroof *et al*. (1984) with slight modifications. The quality and quantity of extracted DNA samples were determined using 0.8% agarose gel electrophoresis and spectrophotometry.

To assess the genetic diversity of genotypes, 30 polymorphic genomic microsatellite primer pairs of Xgwm, Xbarc and Xwmc SSR marker types were selected from different chromosomes based on previous studies and their genome coverage.

A ten-microliter volume, polymerase chain reaction was used according to CIMMYT protocol (Warburton, 2005). Thermal cycling consisted of an initial temperature of 94°C for five minutes, 35 denature cycles at 94°C each for 30 seconds, 30 seconds minute at 50 to 67°C for primer annealing, 40 seconds at a temperature of 72°C and the final extension at 72 ° C for 7 minutes.

Gel electrophoresis and scoring PCR products

PCR products were separated using 6% denature polyacrylamide gel electrophoresis. Polyacrylamide gels were stained with silver nitrate according to ambionet[1].

Band patterns amplified by SSR markers, were scored according to the marker band positions compared to molecular weight marker, where 1 presented the presence, and 0 the lack of a band.

Data Analysis

Data analysis of grain iron content and analysis of variance for the trait data were performed using SPSS and XLSTAT software. The analysis of data obtained from microsatellite bands was performed with NTSYSpc-2.02, XLSTAT and Excel software. Diversity indices including the number of alleles, major allele frequency, and PIC were calculated for each marker using Excel software. Cluster analysis of relationships between genotypes based on SSR data was performed with Dice similarity coefficient and ward method using NTSYSpc-2.02 and XLSTAT software.

To identify markers associated with the grain iron content and ultimately determine the chromosomal regions associated with the trait, stepwise regression analysis was carried out taking into account the grain Fe content data as the dependent and marker data as independent variables using XLSTAT software.

[1] http://www.cimmyt.org/ambionet

Results and discussions

Grain Fe content

After reading the prepared iron standards, extracts prepared from grain samples were read using atomic absorption spectrophotometer, the data were entered in excel software for analyses. The range of iron content in the grains of genotypes was 34-53mg/kg with a mean and standard deviation of 43.57±5.12.

Table 1. The results of ANOVA on genotypes grain iron content using complete randomized block design.

Source of variation	Degree of freedom	The mean of squares of grain iron content (mg/kg)	F
Block	2	491.2	15.2**
Genotype	39	103.4	3.2**
Experimentalerror	78	32.3	

**significant at < 0.01 CV = 9.26 $R^2 = 0.714$

After testing the validity of the assumptions of the statistical model of analysis of variance based on the completely randomized block design, data were analyzed. The results of ANOVA showed a significant block effect at $P<0.01$, confirming the correctness of the experimental design based on the field conditions, it also revealed a significant difference between genotypes for grain iron content at $P<0.01$ (Table 1).

Because of the difference in environmental conditions and also the type of population used in different studies, wheat grain Fe content varies in terms of both range and mean. Monasterio and Graham (2000) estimated the wheat grain iron content in the range of 25 to 73 mg/kg based on a study on 324 selected wheat genotypes in the field conditions. In a study on the Emmer wheat (*Triticum dicoccoides*), in both greenhouse and field conditions, Cakmak *et al*.(2004) have estimated the amount of grain iron content for aforesaid conditions in the range of 15 to 94 and 21to 91mg/kg, respectively. In a study on spring and winter wheat cultivars, Morgounov *et al*. (2007) have reported the range of the grain iron concentration from 65 to 25mg/kg. In another study on 150 hexaploid and 25 tetraploid genotypes performed in field condition in the Europe, Zhao *et al*. (2009) have reported wheat grain iron content in the range of 28.9-51 mg/kg. The range of Fe concentration of bread wheat in the present study was similar to those reported in earlier researches (Oury *et al*., 2006; Morgounov *et al*., 2007; Zhao *et al*., 2009; Badakhshan *et al*. 2013). As mentioned above, ANOVA showed highly significant differences between wheat cultivars for grain Fe concentrations. Studies with rice and wheat, and preliminary studies with wild relatives and landraces of wheat have demonstrated a considerable

variation in grain Fe concentration (Badakhshan *et al*., 2013; Genc *et al*., 2005; Gomez-Becerra *et al*., 2010a, b).

Analysis of microsatellite data

After running and discriminating the PCR products on denature polyacrylamide gels, band patterns were scored according to the marker band positions based on 1, presence, and 0, lack of a band (Figure 1). In order to verify the results of microsatellite and diversity level in the studied population, microsatellite diversity indicators including allele number, polymorphism information content (PIC) and discriminative power (Tables 2 and 3) were calculated and cluster analysis was carried out using Dice dissimilarity coefficient and Ward method as a result of consistency with the pedigree and the origins of genotypes (Figure 2).

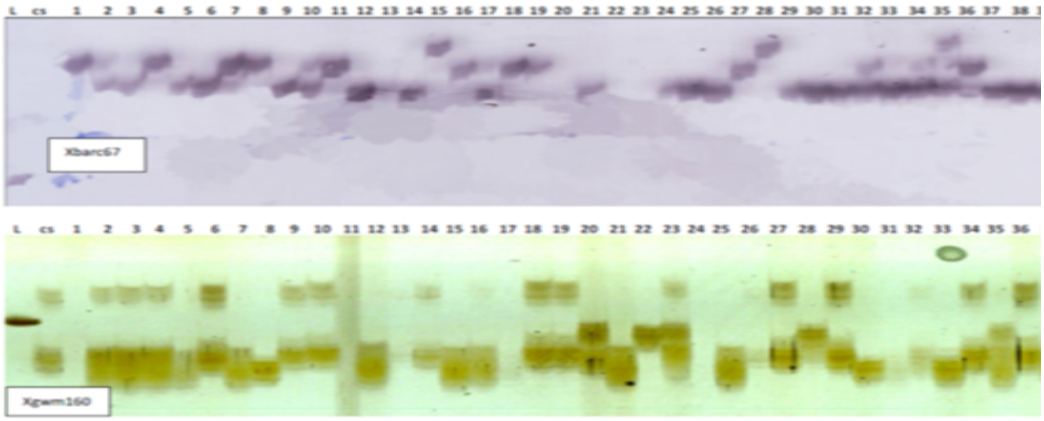

Figure 1. The profile of acrylamide gel band pattern of two SSR markers Xbarc67 (above) and Xgwm160 (below).

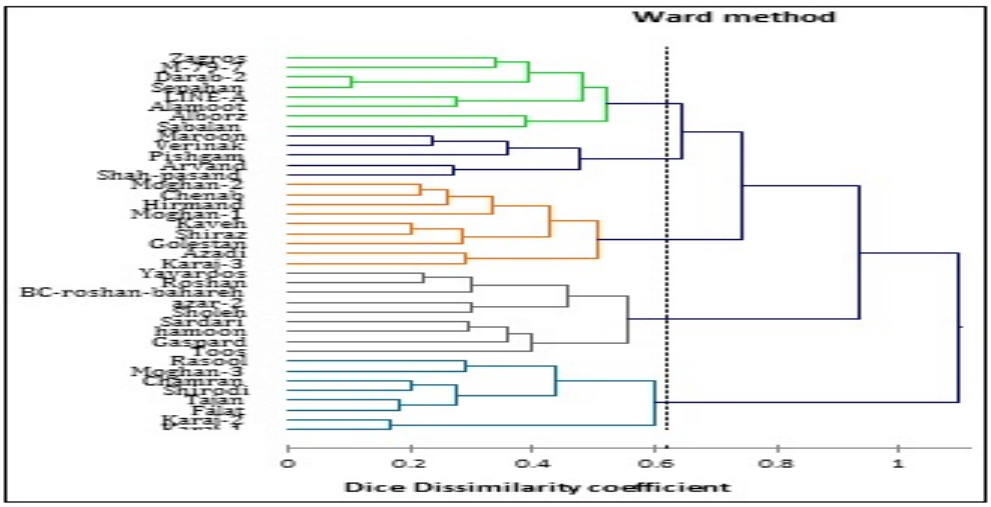

Figure 2. Dendrogram constructed for 38 wheat genotypes using SSR marker.

A number of 2-9 with an average of 4.5 alleles per locus resulted from microsatellite amplification among studied genotypes. The mean of major allele frequency and polymorphic information content (PIC) were 0.56 and 0.55, respectively. Although the main purpose of selecting markers used in the present study was to find genomic regions controlling grain iron content, these markers detected an appropriate level of genetic diversity in the population compared to other studies that aim to study genetic diversity specifically So that, genetic diversity index of three markers Xwmc617, Xbarc146 and Xgwm282 was up to 0.8, (Table 2). Therefore, we can offer these markers to study genetic diversity and kinship in near relatives and to separate such genotypes with high confidence using a fewer number of markers.

Several different studies on genetic diversity were searched and compared together, of which some agreed or closed to the present study. Mohammadi *et al*. (2008) have assessed the genetic diversity of Iranian wheat cultivars using microsatellites and reported the averages of the number of alleles per locus and gene diversity 8.53 and 0.74, respectively. Lee *et al*. (2006) with a study on 48 subspecies of T. *turgidum* in 16 genomic microsatellite loci have identified 96 with an average of 6.1 alleles per locus. In another done on winter wheat, a range of 2-14 with an average of 4.15 alleles per SSR locus and an average of PIC 0.56 have been reported (Wu *et al*. 2012).

The results of cluster analysis have clustered genotypes into five groups. The relationships among genotypes would also confirm the validity of the obtained results from SSR data.

Table 2. Diversity indicators calculated for genomic SSR mark.

Genomic SSRs	Number of alleles	PIC	D
Xbarc29	4	0.47	0.69
Xbarc67	4	0.51	0.69
Xbarc83	4	0.75	0.94
Xbarc146	7	0.81	0.97
Xbarc98	4	0.64	0.78
Xbarc124b	6	0.77	0.88
Xbarc48	3	0.19	0.32
Xgwm3	4	0.59	0.86
Xgwm6	6	0.67	0.93
Xgwm18	3	0.33	0.51
Xgwm149	3	0.63	0.90
Xgwm282	7	0.80	0.96
Xgwm397	3	0.38	0.59
Xgwm400	3	0.61	0.58
Xgwm473	2	0.18	0.31
Xgwm11	2	0.21	0.35
Xgwm46	8	0.77	0.97
Xgwm95	5	0.66	0.92
Xgwm160	4	0.72	0.93
Xgwm219	4	0.69	0.87
Xgwm312	4	0.29	0.52
Xgwm332	6	0.77	0.94
Xgwm368	5	0.31	0.53
Xwmc182	3	0.31	0.51
Xwmc289	4	0.55	0.90
Xwmc617	9	0.84	0.98
Mean	4.5	0.55	0.74
S.D	1.8	0.21	0.23

Correlation analysis between SSR data and grain Fe content

Based on the results of stepwise regression analysis between microsatellite data (independent variable) and the grain Fe content (dependent variable), Xwmc617 (4A-4B-4D) and Xgwm160 (4A) at the level of $P<0.01$ and Xbarc146 (6D-6B-6A) at the level of $P<0.05$ were significantly correlated to the trait. It have been reported in several studies that diploid

and tetraploid species which carry A and B genomes, to be promising containers of grain Fe content and the other microelements correlated to Fe content such as Zn and protein. In a study on recombinant lines resulted from the cross between wild emmer and durum wheat, Peleg*et al*. (2009) have reported 11, 6 and 10 QTLs associated with grain iron content, zinc content and grain protein concentration respectively. The study also reported that two of the QTLs are located on chromosomes 7B and 4B. The marker Xwmc617 used in the present study carrying one of the amplification sites on the short arm of chromosome 4B, showed the most correlation with the wheat grain Fe content from among three aforementioned ones. In another study on A genome of diploid wheat, Tiwari *et al*. (2009) identified two QTLs on chromosomes 2A and 7A associated with grain iron content and another on 7A associated with grain zinc content. In another study, chromosomes 7A, 6B, 2A and 7B have showed a close relationship with grain protein content (Xu *et al*., 2008). With a study on hexaploid wheat Genc *et al*. (2009) reported one QTL related to grain iron content on chromosome 3D and four others on chromosomes 3D, 4B, 6B and 7A, related to grain Zn content, note that the marker Xbarc146 in the mentioned study was also nearby the grain Zn content QTL. Using double haploid lines of two hexaploid wheat cultivars, Shi *et al*. (2008) identified seven QTLs on chromosomes 3A, 4A, 2D and 4D related to grain zinc content and concentration.

Table 3. Diversity indicators calculated for EST SSR markers and total mean and standard deviation calculated for genomic +EST SSRs.

EST SSRs	Number of alleles	PIC	D
edm16	2	0.32	0.51
edm28	4	0.5	0.62
edm96	4	0.57	0.9
edm54	3	0.22	0.38
edm80	5	0.73	0.94
Mean	3.6	0.47	0.67
S.D	1.14	0.2	0.24
Total mean (genomic+EST)	4.35	0.54	0.73
Total S.D (genomic+EST)	1.72	0.21	0.22

According to the results of different studies in this case, it has been demonstrated that di and tetraploid wheat species which possess genomes A and B, contain a significantly higher amount of grain Fe and Zn content and also the percent of protein than hexaploid wheat species (Tiwari 2009; Peleg 2009; Genc 2009; Badkhshan *et al*., 2013). Thus, we can conclude that, chromosomes of genomes A and B have an important role in controlling micronutrients content, such as iron, in wheat grain and the significant correlation between these nutrients in wheat grain, allow the simultaneous improvement of these micronutrients in the wheat grain using traditional breeding techniques with the help of techniques such as marker-assisted selection and also using modern biotechnology techniques.

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ارزیابی تنوع ژنتیکی و شناسایی نشانگرهای SSR دارای پیوستگی با محتوی آهن دانه در ژنوتیپ‌های گندم رایج در ایران

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چکیده

آهن یکی از مهمترین مواد مغذی در جیره غذایی انسان است. با توجه به مصرف بالای مواد غذایی اصلی مانند گندم، کمبود آهن در این محصولات منجر به نارسایی‌های تغذیه‌ای و مشکلات مربوط به آن خواهد شد. برای شناسایی نشانگرهای ریزماهواره دارای پیوستگی با محتوی آهن دانه گندم، ۳۸ ژنوتیپ گندم رایج در ایران با استفاده از ۳۰ جفت نشانگر ریزماهواره ژنومی و EST مورد بررسی قرار گرفتند. نتایج آزمایشات مزرعه‌ای اختلاف معنی‌داری را از نظر محتوی آهن دانه بین ژنوتیپ‌های مورد مطالعه نشان داده و محتوی آهن دانه در ژنوتیپ‌ها در دامنه ۳۴ تا ۵۳ میلی‌گرم بر کیلوگرم بود. در آزمایشات مولکولی، تعداد آلل به ازای جایگاه ریزماهواره در دامنه ۲ تا ۹ با میانگین ۴/۵ و میانگین محتوای اطلاعات چند شکلی (PIC) ۰/۵۵ برآورد شد. برای برآورد ارتباط بین نشانگرهای ریزماهواره و محتوی آهن دانه از تجزیه رگرسیون گام به گام استفاده شد. نتایج نشان داد که نشانگرهایXwmc617 (4A, 4B, 4D)، Xgwm160 (4A) و Xbarc146 (6D, 6B, 6A) دارای همبستگی معنی‌داری با محتوی آهن دانه گندم بودند. نتایج این پژوهش می‌تواند در مطالعات گسترده‌تر از جمله اصلاح به کمک نشانگر در گندم برای افزایش محتوی آهن دانه مورد استفاده قرار گیرد.

کلمات کلیدی: ریزمغذی، نشانگر ریزماهواره، رگرسیون گام به گام، گندم.