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Haplotype analysis of molecular markers linked to QTLs controlling Iron content in rice grains

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Abstract

Iron deficiency affects 2 billion people currently and the number is increasing. Biofortification of rice is one of the best approaches for solution this problem. Molecular marker techniques can greatly improve the efficacy of breeding programs to improve grain iron content and bioavailability in major staple crops such as rice. In the current study, the haplotype variation of three loci controlling iron content was evaluated using 50 genotypes and 14 associated microsatellite markers. The grain iron content analysis of a collection of 50 rice genotypes showed large variation from 9.06 (Sepidrud cultivar) to 50.55 (Mehr cultivar) $mg.kg^{-1}$ indicating the existence of genetic potential to increase the content of this micronutrient in rice grain. Based on the results of genotyping RM276 indicated highest polymorphism information content (0.83). The haplotype diversity analysis showed, allelic pattern of 132-176-200 bp in the Norin22 and Shahak cultivars on chromosome 12 had the most similarity with haplotype of reference cultivar Mehr. This allele combination can be informative markers for improvement of iron content in rice grains through marker-assisted selection programs. Furthermore, the presence of allele combinations, different from reference haplotype in Dadras and Farideh cultivars (with high iron contents) indicated the presence of novel source of QTLs controlling grain iron content in Iranian rice cultivars.

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Introduction

Iron deficiency is one of the most prevalent micronutrient deficiencies in humans, causing 0.8 million deaths annually and affecting approximately two billion (World Health Organization 2002, http://www.who.int/whr/2002/). The development of new cereal varieties containing increased concentrations of iron and other essential micronutrients, an approach known as biofortification, offers an inexpensive and sustainable solution to the iron deficiency problems (Johnson *et al.*, 2011).

Rice serves as staple food for more than half of the world's population. In developing countries, rice accounts for 715 Kcal/capita per day; 27% of dietary energy supply, 20% of dietary protein, and 3% of dietary fat (FAO statistical databases 2001, http://faostat3.fao.org/home/E). Rice has been found to contain starch, proteins, and vitamins (Ensminger *et al.*, 1995); yet the largest nutritional deficiency occurring globally and among rice-consuming countries is mineral deficiency (Fe, I, Zn), vitamin, and protein-energy malnutrition. Thus, increasing the iron content of rice grain has great potential in combating iron deficiency and will have a dramatic impact on human health (Guerinot, 2001; Clemens *et al.*, 2002). Traditional breeding, maker-assisted breeding, and plant transformation techniques and a combination of these techniques can be further exploited to mitigate the iron deficiency in rice and humans. Knowledge on the gene action of a trait is essential to determine the best breeding strategy to incorporate the trait of interest into breeding populations. Gregorio *et al.* (2000) reported that the type of genetic variation and its relative importance for grain iron content varied from one breeding population to another. Dominant and epistatic effects for iron content in rice grain were observed. Significant environmental variances suggested that rice grain iron content could be modified, to some extent, by the growing environment. The results showed that the rice grain iron content is a quantitative trait. If these micronutrients could be incorporated through breeding in a staple food crop such as rice, expenditures for a micronutrient intervention program could decrease markedly (Gregorio and Htut, 2003). Quantitative trait locus (QTL) mapping is a powerful tool for understanding the genetic controls underlying complex traits (Yano and Sasaki, 1997; Yamamoto *et al.*, 2009). Several QTLs for grain micronutrient content have been identified and mapped on rice chromosomes using molecular markers (Stangoulis *et al.*, 2007; Lu *et al.*, 2008; Susanto, 2008; Ishikawa *et al.*, 2010; Anuradha *et al.*, 2012). Anuradha *et al.* (2012) identified some QTLs for iron and zinc in rice grain using a population of 168 F_6 recombinant inbred lines (RILs). Genome wide mapping showed five QTLs on chromosomes 1, 3, 5, 7 and 12 significantly linked to iron, zinc or both. QTLs for iron were co-located with QTLs for zinc on chromosomes 7 and 12. Stangoulis *et al.* (2007) mapped three QTLs associated with iron concentration on chromosomes 2, 8 and 12, explaining approximately 17%, 18% and 14% of the total phenotypic variation, respectively. Susanto (2008) reported two QTLs controlling iron content in rice grains, were found on chromosomes 3 and 6. These were explained 17.2% and 12.3% of the total variation, respectively.

In recent times there have been advances in development and mapping of QTLs controlling iron content in rice. However, there are no reports on evaluation of haplotype variation of iron QTLs in Iranian rice cultivars and other genotypes by reported linked molecular markers.

In this study, it has been evaluated genomic regions encompassing three known QTLs controlling iron content that located on chromosome 3, 6 and 12 in rice by microsatellite or Simple Sequence Repeat (SSR) markers (Stangoulis *et al.*, 2007; Susanto, 2008; Ishikawa *et al.*, 2010; Anuradha *et al.*, 2012).
The objectives of the study were to: (1) Assess genetic diversity for grain iron content in a rice germplasm, (2) identify suitable parents to exploit in breeding programs to enhance levels of grain iron content, (3)

analyze haplotype diversity of markers linked to three previously identified QTLs controlling iron in rice grains with the aim of identifying and classifying allelic variation at these loci. The haplotype data presented here will provide the basis from which breeders may accurately identify, utilize and trace allelic variation at these loci in their breeding material (4) identify and utilize of novel alleles or allele combinations not previously deployed.

Materials and methods

Plant materials

A total of 50 rice genotypes were obtained from the Rice Research Institute of Iran (RRII) (Table 1). Genotypes used included 26 Iranian landrace cultivars and 15 Iranian improved genotypes, 5 rice genotypes with different origin and 4 IRRI rice lines. All genotypes were grown in the same soil and season. The grain iron content of all the genotypes was measured with two replications using dry ash method and atomic absorption spectrophotometry (Munson *et al.*, 1990).

Genomic DNA extraction and SSR assay

Template DNA was extracted using a modified CTAB method (Saghai-Maroof *et al.*, 1984). SSR markers were chosen on the basis of their proximity to QTL regions associated with grain iron content and genome specificity. The SSRs were both public and proprietary databases of SSRs from Gramene (http://www.gramene.org) and Rice Togo Browser (http://agri-trait.dna.affrc.go.jp). Fourteen SSR markers were applied to a subset of 50 rice genotypes (Table 2). The polymerase chain reaction (PCR) was conducted in a 15 µL volume using the Applied Biosystems Veriti thermocycler. Each 15 µL reaction contained 20 ng of genomic DNA, 1.5 µL of 10 × PCR buffer, 0.5 µL of 50 mM $MgCl_2$,0.18 µL of 10 mM dNTP, 0.4 µL of each SSR primer (5 µM) and 0.15 µL of 5U/µL *Taq* DNA polymerase. The PCR amplification was performed according to the cycle profile: initial denaturation at 94 °C for 3 min and then 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at the temperature depending on the marker used (54-58 °C), 1 min extension at 72 °C and 7 min at 72 °C for the final extension. Amplicons were electrophoresed through a 3% MetaPhor agarose gel, and stained with ethidium bromide.

Data analysis

Analysis of variance for grain iron content data were performed using SPSS statistical software (version 16.0). The size of amplified fragments was determined by comparing the migration distance of amplified fragments relative to the molecular weight of known size markers, 100 bp DNA ladder using Alpha-Ease FC 5.0 software (Alpha Innotech, USA). PowerMarker ver 3.25 software was used to calculate the average number of alleles, major allele frequency, gene diversity, and polymorphism information content (PIC) values (Liu and Muse, 2005). Haplotype diversity was analyzed according to McCartney *et al.* (2004) and Mohammadi-Nejad *et al.* (2010).

Results

The grain iron content of cultivars varied from 9.06 (Sepidrud cultivar) to 50.55 (Mehr cultivar) $mg.kg^{-1}$, and the average of iron content was estimated 20.33 $mg.kg^{-1}$. The analysis of variance results showed highly significant difference among the genotypes for iron content. Furthermore, an average of iron content in landraces cultivars (22.82 $mg.kg^{-1}$) were significantly higher than improved cultivars (17.20 $mg.kg^{-1}$).

The 14 SSR markers revealed 71 alleles among the 50 rice genotypes. The number of microsatellite alleles varied from 2 to 9, which RM276 produced the highest number of alleles and RM28722 produced the lowest (Table 2). PIC value ranged from 0.28 to 0.83, the highest value belonged to RM276 and RM28722 showed the lowest PIC value. Therefore, the SSR marker RM276 was found to be suitable for analysis of genetic diversity among the markers in this research.

In evaluation of haplotype diversity, Mehr cultivar

with the highest iron content used as reference genotype. Haplotypes were sorted for each QTL by the size of their fragments. Allele combinations for each QTL were compared to the haplotype of reference genotype and similar allele combinations were grouped together. Three QTL regions were analyzed for haplotype diversity.

Table 1. The names of evaluated genotypes in this research and iron averages (mg.kg^{-1}).

Genotype	Iron average	Genotype	Iron average
Iranian landrace genotypes		Iranian improved genotypes	
Dadras	49.93	Mehr	50.55
Sadri	34.32	Gil1	25.85
Farideh	34.20	Bojar	25.08
Deilamani	29.41	Haraz	21.09
Shahak	29.17	Line 29 [from Mohammadi×(Amol3×Tarom)]	19.88
Hashemi	26.03	Neemat	17.51
Abjibuji	25.47	Line 27 [from Asgari× Ch21]	17.43
Mosa tarom	24.98	Gil3	16.43
Hassani	23.12	111	16.12
Cheli	22.94	Fajr	14.94
Hasani fumani	21.91	Dorfak	14.83
Champa budar	21.58	Khazar	14.03
Domsorkh	21.53	Amol3	11.77
Sangejo	21.22	Dasht	9.66
Domsiah	20.55	Sepidrud	9.06
Ghanbarak	20.27	IRRI lines	
Ramezanali tarom	20.24	IR30	18.95
Ghashange	19.21	IR24	12.67
Anbarbu	17.61	Line 18 [from IRRI]	16.51
Rashti sard	16.89	Line 13 [from IRRI]	11.12
Hassan molaeii	16.78	Genotypes with different origin	
Shahpasand	16.43	Norin22	30.82
Aghaeii seiah	16.22	Century patna	13.91
Ali kazemi	15.6	Zinet	13.21
Gharib	14.24	Fuji minori	12.15
Salari	13.47	Onda	9.81

The QTL region on chromosome 6

To study the haplotype diversity of this QTL, sizes of PCR fragments at six SSR markers were determined for all the genotypes. Allelic pattern (162-155-135-357-326-205 bp) was observed in Mehr at six SSR loci: RM217, RM276, RM402, RM19675, RM19708 and RM8226, respectively. Based on allelic pattern of this region, genotypes arranged in various haplotype groups. Used SSR markers for this chromosomal region produced 19 different haplotype groups (Table 3). None of the 50 genotypes showed Mehr haplotype in this QTL but comparison of these genotypes with Mehr pattern showed Norin22 cultivar, with high level of grain iron content, amplified the same SSR alleles (135-326-205 bp) for RM402, RM19708 and RM8226, respectively, that common allele combinations can be important to controlling iron content. Fourteen genotypes allocated to 14 single haplotypes and nineteen genotypes did not have any common alleles with the Mehr haplotype (haplotype No. 19). There are different alleles of the haplotypes reference such as 123 bp for marker RM402 (haplotype No. 19) in most cultivars with high iron content, such as a Dadras, Farideh, Mosa tarom, Sadri and Deilamani could confirmed the role of this allele in controlling the iron content of grains Iranian cultivars.

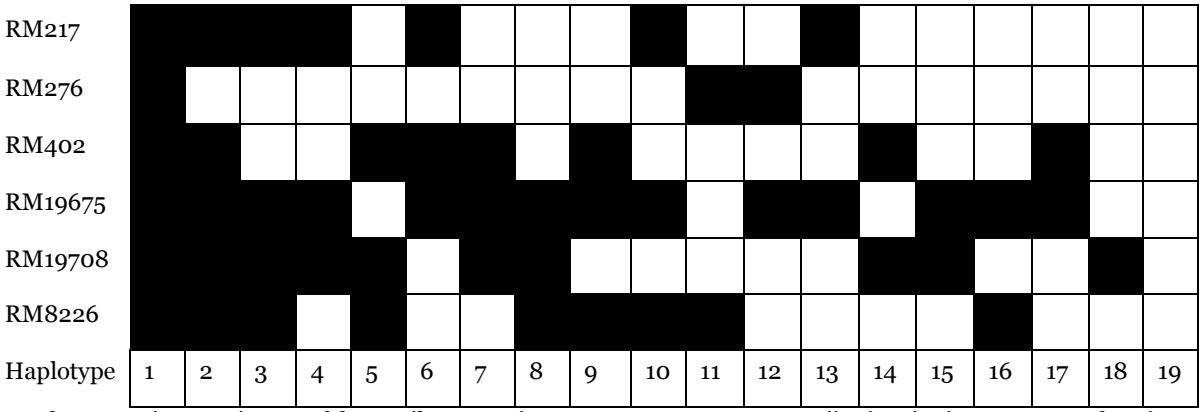
Table 2. List of SSR markers used, including chromosome location, number of amplified alleles, polymorphic information content (PIC) and gene diversity.

Marker	chr	Major Allele Frequency	Allele NO	Gene diversity	PIC	Amplicon size range (bp)
RM6931	3	0.41	5	0.66	0.59	180-233
RM6832	3	0.46	6	0.72	0.69	136-186
RM217	6	0.42	5	0.69	0.64	129-162
RM276	6	0.28	9	0.85	0.83	96-155
RM402	6	0.18	7	0.84	0.82	116-139
RM19675	6	0.86	4	0.25	0.24	357-417
RM19708	6	0.46	3	0.63	0.56	316-337
RM8226	6	0.26	6	0.80	0.78	205-250
RM17	12	0.72	5	0.45	0.42	154-184
RM235	12	0.26	6	0.80	0.78	102-137
RM270	12	0.5	3	0.62	0.54	104-111
RM3331	12	0.31	5	0.78	0.74	128-144
RM1999	12	0.34	5	0.77	0.73	170-227
RM28722	12	0.78	2	0.34	0.28	183-200
Mean		0.45	5.1	0.66	0.62	

The QTL region on chromosome 12

Six polymorphic SSR markers were used for haplotyping of this locus on chromosome 12 using 50 rice genotypes. The genotypes possessed different marker alleles in the same genomic region and Twenty two haplotypes were identified among the 50 rice genotypes (Table 4). Six putative alleles (132-108-133-176-200-154 bp) were presented in Mehr at six microsatellite loci: RM3331, RM270, RM235, RM1999, RM28722 and RM17, respectively. Among them, RM3331, RM1999 and RM28722 amplified 132-176-200 bp PCR fragments in the Norin22 and Shahak cultivars (haplotypes No. 2 and 3) with high iron content, indicating that is explanatory of importance of this QTL. Furthermore, the presence of Dadras, Farideh and Bojar cultivars with high iron content in haplotype No 22 indicated the iron content in these cultivars can be controlled by other new regions.

Table 3. Rice haplotypes produced by SSR markers located on QTL controlling grain iron content on chromosome 6 with reference to Mehr cultivar*.

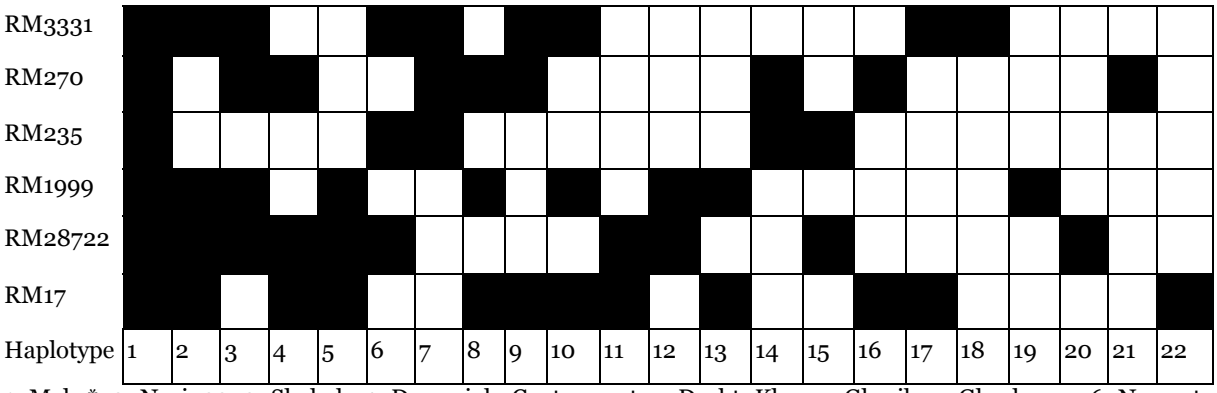


1: Mehr*, 2: Fajr, 3: Bojar, Dorfak, 4: Gil3, 5: Norin22, 6: Century patna, 7: Fuji minori, Line 13, 8: Amol3, Zinet, 9: Sepidrud, 10: Line 27, 11: Cheli, 12: Haraz, 13: Gil1, 14: Hassani fumani, 15: Hashemi, Champa budar, 111, Ghanbarak, Anbarbu, Rashti sard, Aghaii seiah, IR30, Line 18, 16: Dasht, 17: IR24, 18: Shahak, Khazar, Onda, 19: Dadras, Sadri, Farideh, Deilamani, Abjibuji, mosa tarom, Hassani, Domsorkh, Sangejo, Ramezanali tarom, Domseiah, Line 29, Ghashange, Neemat, Hassan molaeii, Salari, Gharib, Ali Kazemi, Shahpasand

The QTL region on chromosome 3

Microsatellite markers for this chromosomal region amplified various sizes of PCR fragments in the genotypes (Table 5). Used microsatellite markers for this chromosomal region produced 4 different haplotype groups. Allelic pattern of Mehr cultivar was 230-186 bp for RM6931 and RM6832, respectively. The QTL region requires that to be investigated with more of SSR markers.

Table 4. Rice haplotypes produced by SSR markers located on QTL controlling grain iron content on chromosome 12 with reference to Mehr cultivar*.



1: Mehr*, 2: Norin22, 3: Shahak, 4: Domseiah, Century patna, Dasht, Khazar, Gharib, 5: Ghashange, 6: Neemat, 7: IR24, 8: Abjibuji, 9: Deilamani, 10: Zinet, 11: Cheli, Hassani fumani, Sadri, Ali Kazemi, 12: Line 29, 13: Line 27, Fuji minori, 14: Gil3, Line 13, Line 18, 15: Fajr, Sepidrud, 16: Dorfak, Amol3, Gil1, Hashemi, 111, Ghanbarak, mosa tarom, Hassani, Sangejo, Hassan molaeii, Salari, 17: Anbarbu, 18: IR30, Shahpasand, 19: Champa budar, 20: Onda, 21: Haraz, 22: Bojar, Rashti sard, Aghaii seiah, Dadras, Farideh, Domsorkh, Ramezanali tarom.

Discussion

Fe deficiency severely impairs plant growth and is a widespread human dietary problem, with particularly high numbers of affected children and females. Understanding the genetic basis of accumulation of micronutrients in the grains will provide the basis for devising the plant breeding strategies and for improving grain micronutrient content through marker assisted selection (Tiwari *et al.*, 2009). So far, many studies assessed grain iron content in rice germplasm and reported broad range for iron content in rice grains. Gregorio *et al.* (2000) reported from preliminary studies at IRRI that variation of grain iron content existed in rice and was large enough to undertake breeding for enhanced high iron content in rice grains. Similar results were also reported by Welch and Graham (2004) from the evaluation of nearly 7000 samples. The iron content ranged from 6.3 ppm to 24.4 ppm with a mean value of 12.2 ppm in brown rice. Popular cultivars contained about 12 ppm iron. Some traditional varieties have double these amounts. Similarly, Anandan *et al.* (2011) evaluated 202 rice genotypes and reported that iron concentration ranged from 1.25 to 39.19 $mg.kg^{-1}$ with a mean value of 15.84 $mg.kg^{-1}$ in traditional genotypes and 4.10 to 20.64 $mg.kg^{-1}$ with a mean value of 10.95 $mg.kg^{-1}$ in improved genotypes. In present study, the average of grain iron content (20.33 $mg.kg^{-1}$) was higher than that was reported for 202 rice genotypes. The difference might be due to variation of genotypes and genotype × environment interaction. Similar to this result, Anandan *et al.* (2011) and Gregorio *et al.* (2000) indicated that most of the traditional varieties contained high iron and zinc in the grain, whereas the modern-released varieties produced lower micronutrient content in the grain. These observations can be attributed to the fact that direct selection for high iron content was not part of the previous rice breeding program and the variability in improved varieties for grain iron content was only as a consequence of random drift or indirect selection effects.

In the current study, among the twenty-three haplotypes identified for the iron QTL on chromosome 6, haplotype of Norin 22 cultivar (contained high iron) showed same SSR alleles with Mehr haplotype. Based on the result it is concluded RM402, RM19708 and RM8226 markers by allelic pattern of 135-326-205 bp are helpful allele combinations for identifying and utilizing iron sources in breeding programs. Further, some genotypes such as Dadras, Sadri, Farideh, Deilamani, Abjibuji, Mosa tarom had distinct haplotype patterns in this QTL, well-known iron sources, suggesting that they could carry new iron QTLs. Therefore, these cultivars could potentially be exploited to identify new QTLs of iron grain in Iranian rice.

Table 5. Rice haplotypes produced by SSR markers located on QTL controlling grain iron content on chromosome 3 with reference to Mehr cultivar*.

RM6931				
RM6832				
Haplotype No	1	2	3	4

1: Mehr*, 2: Abjibuji, Haraz, Domseiah, Ghanbarak, Khazar, Zinet, Amol3, Anbarbu, IR30, Onda, Century patna,Rashti sard, 3: Hassani, Cheli, Hassani fumani, Fuji minori, Aghaii seiah, 4: Other genotypes

So far, many studies assessed haplotype variation of genomic regions controlling numerous traits in cereals, but were not found any researches for haplotype diversity of micronutrients QTLs. Mohammadi-Nejad *et al.* (2010) studied haplotype diversity for saltol QTL controlling salinity tolerance in rice, they found total of 16 haplotype groups using 8 microsatellite markers and 30 genotypes. Ogbonnaya *et al.* (2011) evaluated haplotype diversity of preharvest sprouting QTLs in the 28 wheat genotypes and identified the haplotypes associated with the different genotypes at the studied PHS-resistant QTLs. Also McCartney *et al.* (2004) identified 76 haplotypes among the 79 wheat lines for fusarium head blight resistance QTLs.

It is concluded that Mehr and Norin 22 cultivars and some genotypes belong to different haplotypes, such as Dadras and Farideh cultivars, can be used for the genetics and breeding programs for improvement of iron content in rice grains.

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