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# Haplotype diversity of microsatellite markers linked to QTLs controlling zinc content in rice grains

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**Key words:** Allele combinations, Rice grain, SSR marker, Zinc.

**Abstract**

Zinc deficiency is one of the serious problems of malnutrition in developing countries. Identifying genotypes with high levels of grain zinc in rice genetic resources and understanding the genetic basis of its accumulation is a prerequisite in genetically breeding programs of zinc. In this study, the haplotype diversity of three loci controlling zinc content were evaluated using 50 genotypes and 14 associated microsatellite markers. The grain zinc content was measured using atomic absorption with two replications. The Neemat and Dadras cultivars with the amount of 10.94 and 36.92 $mg.Kg^{-1}$ had the lowest and highest grain zinc content, respectively. According to the analysis of molecular, RM6925 was showed highest polymorphism information content (0.79). Haplotype of Mehr cultivar with allele combinations of 166-184-327-266 bp on chromosome 8 had the most similarity with haplotype of reference cultivar Dadras. This allele combination can be regarded as the effective combination of alleles for marker-assisted selection in the breeding programs of this microelement. Furthermore, the presence of allele combinations different from reference haplotype in cultivars with high zinc concentrations indicated the presence of new QTLs controlling grain zinc content in these rice cultivars.

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**Introduction**

Rice is an indispensable staple food for half of the world's population. In countries where rice is used as staple food, the per capita consumption is very high ranging from 62 to 190 kg/year (Graham *et al.,* 1999; Chandel *et al.,* 2011). However, rice is a poor source of essential minerals such as zinc and iron (Bouis and Welch, 2010).

Zinc deficiency is serious nutritional problem for humans and is particularly prevalent among children and pregnant women, especially in developing countries (World Health Organization 2002, http://www.who.int/whr/2002/). Therefore, increasing the zinc content and bioavailability of cereals such as rice grains is an important target for breeders and offers the potential benefits to a large proportion of human population (Zhu *et al*., 2007; Cakmak, 2008; Palmgren *et al*., 2008). Traditional breeding, maker-assisted breeding, and plant transformation techniques and a combination of these techniques can be further exploited to mitigate the zinc deficiency in rice and humans. Many researchers have already evaluated genetic diversity for zinc content in cereal grains such as rice (Gregorio *et al*., 2000; Zhang *et al*., 2004), wheat (Cakmak *et al*., 2000; Ortiz-Monasterio and Graham, 2000; Balint *et al*., 2001) and reported the narrow genetic base for zinc in cultivated rice (Gregorio *et al*., 2000; Ortiz-Monasterio and Graham, 2000; Stangoulis *et al*., 2007).

Zinc content is quantitative trait which is affected by the environment (Gregorio *et al*., 2000; Virk *et al*., 2006, 2007). 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). QTL mapping has been used extensively to identify genetic loci determining mineral content in rice (Ishikawa *et al*., 2005; Lu *et al*., 2008). Stangoulis *et al.* (2007) identified some QTLs for Fe, Zn, Mn and P in rice grain using a doubled-haploid population. Zinc content had two QTLs on chromosomes one and 12, explaining 15% and 13% of the total phenotypic variation, respectively. The zinc QTL on chromosome 12 was co-located with the iron QTL. Garcia-Oliveira *et al.* (2009) reported three QTLs controlling zinc content in rice were mapped on chromosomes 5, 8, and 12. The QTL near marker RM152 on chromosome 8 accounted for 19% of phenotypic variation and the QTL that was located on chromosome 12 accounted for 9% of phenotypic variation. Susanto (2008) mapped two QTLs associated with zinc content in polished rice grain were detected on chromosomes 6 and 12.

In recent times there have been advances in development and mapping of QTLs controlling zinc content in rice. However, there are no reports on evaluation of haplotype variation of zinc 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 zinc content that located on chromosome 6, 8 and 12 in rice by microsatellite or Simple Sequence Repeat (SSR) markers (Stangoulis *et al*., 2007; Susanto, 2008; Garcia-Oliveira *et al*., 2009; Ishikawa *et al*., 2010).

The objectives of this research were to: (1) identify genotypes with high zinc content from a rice germplasm to exploit in breeding programs to enhance levels of zinc content, (2) investigate genetic relationships of rice genotypes with same zinc content, (3) analyze haplotype diversity of markers linked to three previously identified QTLs controlling zinc 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*

Plant materials of this study were 50 different rice genotypes, including 26 Iranian landrace cultivars and 15 Iranian improved genotypes, 5 rice genotypes with different origin and 4 IRRI rice lines, which were received from Rice Research Institute of Iran (RRII) (Table 1). All genotypes were grown in the same soil and season. The grain zinc content of all the genotypes was measured with two replications using dry ash method and atomic absorption spectrophotometry (Munson *et al.*, 1990).

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

| Genotype | Zinc average | Genotype | Zinc average |
|---|---|---|---|
| Iranian landrace genotypes | | Iranian improved genotypes | |
| Dadras | 36.92 | Bojar | 34.30 |
| Abjibuji | 36.69 | Mehr | 33.19 |
| Hassan molaeii | 28.38 | Haraz | 22.17 |
| Deilamani | 26.81 | 111 | 19.85 |
| Shahak | 25.83 | Gil1 | 18.85 |
| Sangejo | 25.57 | Line 29 [from Mohammadi×(Amol3×Tarom)] | 17.82 |
| Aghaeii seiah | 24.67 | Gil3 | 16.37 |
| Hassani | 23.59 | Line 27 [from Asgari× Ch21] | 15.63 |
| Champa budar | 21.78 | Dorfak | 15.53 |
| Ghashange | 21.32 | Khazar | 15.04 |
| Cheli | 21.06 | Sepidrud | 13.18 |
| Gharib | 20.42 | Fajr | 13.13 |
| Ghanbarak | 20.29 | Amol3 | 12.87 |
| Hasani fumani | 19.85 | Dasht | 11.46 |
| Mosa tarom | 19.36 | Neemat | 10.94 |
| Rashti sard | 19.08 | IRRI lines | |
| Ramezanali tarom | 18.85 | Line 18 [from IRRI] | 21.58 |
| Ali kazemi | 18.85 | Line 13 [from IRRI] | 17.74 |
| Domsorkh | 18.13 | IR30 | 17.35 |
| Shahpasand | 17.33 | IR24 | 13.85 |
| Salari | 16.71 | Genotypes with different origin | |
| Farideh | 16.12 | Fuji minori | 15.40 |
| Sadri | 16.07 | Onda | 20.01 |
| Anbarbu | 15.76 | Zinet | 14.75 |
| Domsiah | 15.68 | Norin22 | 18.98 |
| Hashemi | 11.25 | Century patna | 17.48 |

*DNA extraction, SSR genotyping*

Template DNA for the polymerase chain reactions (PCR) was extracted from leaf tissue using the modified CTAB method (Saghai-Maroof *et al.*, 1984). SSR markers were chosen on the basis of their proximity to QTL regions associated with zinc 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). A set of 14 SSR

assays was applied to a subset of 50 rice genotypes (Table 2). The PCR reaction was performed in a 15 µL volume using the Applied Biosystems Veriti thermocycler. Each 15 µL reaction contained 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), 0.15 µL of 5U/ µL *Taq* DNA polymerase and 20 ng of genomic DNA. The amplification program consisted of an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 54-58 °C for 1 min, 72 °C for 1 min, and ending with a 72° C for 7 min final extension. Amplicons were electrophoresed through a 3% MetaPhor agarose gel, and stained with ethidium bromide.

**Table 2.** List of SSR markers used, 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) |
|---|---|---|---|---|---|---|
| RM8226 | 6 | 0.26 | 6 | 0.80 | 0.78 | 205-250 |
| RM7193 | 6 | 0.5 | 8 | 0.69 | 0.65 | 122-186 |
| RM152 | 8 | 0.38 | 5 | 0.71 | 0.66 | 132-159 |
| RM337 | 8 | 0.46 | 5 | 0.69 | 0.64 | 154-457 |
| RM6925 | 8 | 0.32 | 10 | 0.81 | 0.79 | 155-287 |
| RM407 | 8 | 0.44 | 4 | 0.69 | 0.64 | 162-176 |
| RM22253 | 8 | 0.42 | 3 | 0.64 | 0.56 | 306-327 |
| RM22254 | 8 | 0.58 | 4 | 0.57 | 0.5 | 252-274 |
| 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 | 0.67 | 0.62 | |

*Data analysis*

Analysis of variance for zinc content data were performed using SPSS program. 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 version 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 zinc content of cultivars varied from 10.94 (Neemat cultivar) to 36.92 (Dadras cultivar) $mg.kg^{-1}$ (Table 1), and the average of grain zinc content was estimated 19.68 $mg.kg^{-1}$. The analysis of variance results showed highly significant difference among the genotypes for grain zinc content and an average of grain zinc content in landraces (21.40 $mg.kg^{-1}$) were significantly higher than improved cultivars (17.81 $mg.kg^{-1}$).

The fourteen SSR markers revealed 71 alleles among the 50 rice genotypes. The number of microsatellite alleles varied from 2 to10, which RM6925 produced the highest number of alleles and RM28722 produced the lowest (Table 2). PIC value ranged from 0.28 to

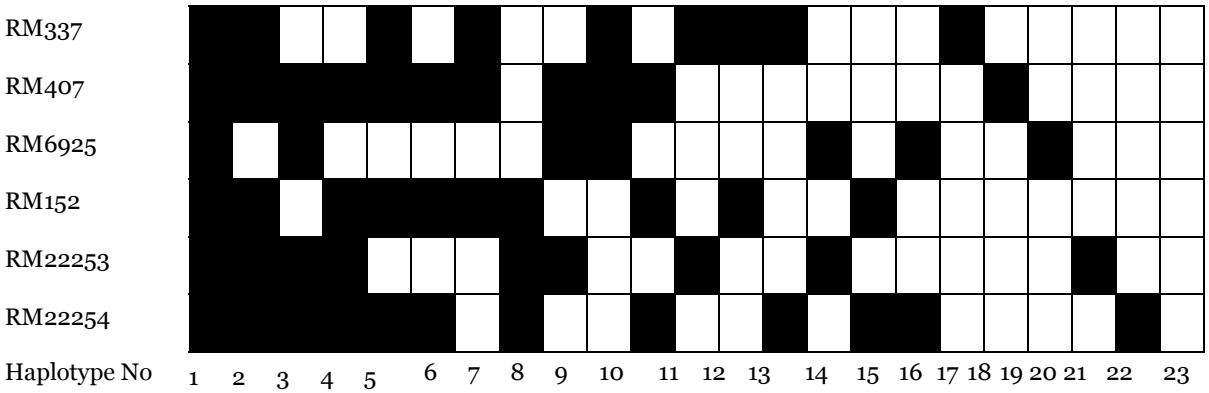
0.79, the highest value belonged to RM6925, while RM28722 showed the lowest PIC value. Therefore, the SSR marker RM6925 was found to be suitable for analysis of genetic diversity among the markers in this research.

In evaluation of haplotype diversity, Dadras cultivar with the highest grain zinc 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.

*The QTL region on chromosome 8*

It has been employed six SSR markers to haplotyping this locus in our genotypes. Allelic pattern (190-166-184-159-327-266 bp) was observed in Dadras at six SSR loci: RM337, RM407, RM6925, RM152, RM22253 and RM22254, respectively. Based on allelic pattern of this region, genotypes arranged in various haplotype groups .Twenty-three haplotypes were identified among the 50 rice genotypes (Table 3). None of the 50 genotypes showed Dadras haplotype in this QTL but haplotypes No. 2, 3, 4 and 5 were similar to Dadras haplotype in most SSRs.

**Table 3.** Rice haplotypes produced by SSR markers located on QTL region associated with zinc content on chromosome 8 with reference to Dadras cultivar*.




1: Dadras*, 2: Dasht, 3: Mehr, 4: Gil3, 5: Dorfak, 6: Haraz, 7: Bojar, 8: Line 18, 9:Rashti sard, Sepidrud, 10: Line 29, 11: Line 27, 12: Aghaii seiah, 13: Khazar, Neemat, 14: Fajr, Amol3, Norin22, 15: Hassani, Gil1, Line 13, 16: Zinet, IR30, 17: Century patna, 18: Ghashange, Cheli, Hassani fumani, 19: Onda, Anbarbu, 20: Ghanbarak, 21: Abjibuji, Hassan molaeii, Deilamani, Shahak, Sangejo, Champa budar, mosa tarom, Ramezanali tarom, Shahpasand, Farideh, Fuji minori, 22: IR24, 23: Other genotypes.

Comparison of these genotypes with Dadras pattern showed Mehr cultivar, with high level of grain zinc content, amplified the same SSR alleles (166-184-327-266 bp) for RM407, RM6925, RM22253 and RM22254, respectively, that common allele combinations can be important to controlling grain zinc content. Thirteen genotypes allocated to 13 single haplotypes and eight genotypes did not have any common alleles with the Dadras haplotype (haplotype No. 23).

Haplotype No. 21 which contained only one common marker alleles with Dadras haplotype, included genotypes with high level of grain zinc content such as Abjibuji, Hassan molaeii, Deilamani, Sangejo, Shahak and champa budar (Table 4). Therefore, these genotypes might have different responsible genes for improving grain zinc content. In addition, Abjibuji and Hassan molaeii cultivars demonstrated the same allelic pattern completely in this haplotype.

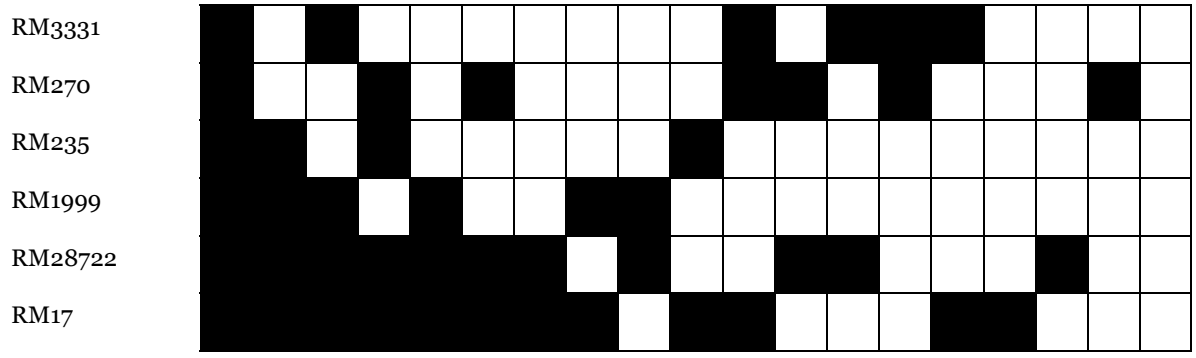
**Table 4.** Allele combinations of some cultivars in QTL region of chromosome 8 (haplotype No. 21).

| Marker | Rice cultivars | | | | | |
|---|---|---|---|---|---|---|
| | Abjibuji | Hassan molaeii | Deilamani | Shahak | Sangejo | Champa budar |
| RM337 | 154 | 154 | 154 | 154 | 154 | 154 |
| RM407 | 162 | 162 | 162 | 171 | 162 | 162 |
| RM6925 | 166 | 166 | 166 | 166 | 166 | 166 |
| RM152 | 141 | 141 | 141 | 132 | 136 | 132 |
| RM22253 | 327 | 327 | 327 | 316 | 327 | 327 |
| RM22254 | 252 | 252 | 259 | 274 | 252 | 274 |
| **Grain Zinc content ($mg.kg^{-1}$)** | 36.69 | 28.38 | 26.81 | 25.82 | 25.56 | 21.78 |

*The QTL region on chromosome 12*

To study the haplotype diversity of this QTL, sizes of PCR fragments at six SSR markers were determined for all the genotypes. Used SSR markers for this chromosomal region produced 19 different haplotype groups (Table 5). Comparison of haplotypes with Dadras haplotype showed, Aghaeii seiah cultivar (haplotype No. 4) with high grain zinc content amplified the same PCR fragments with Dadras (102 and 111 bp) for RM270 and RM235, respectively. Neemat and Sepidrud cultivars which showed the lowest grain zinc content, did not contained any common marker alleles with Dadras haplotype (haplotype No. 19). Furthermore, the presence of Shahak cultivar with high grain zinc content in haplotype No 19 indicated the grain zinc content in this cultivar can be controlled by other new regions.

**Table 5.** Rice haplotypes produced by SSR markers located on QTL region associated with zinc content on chromosome 12 with reference to Dadras cultivar*.




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

*The QTL region on chromosome 6*

Two markers, RM8226 and RM7193, were used for haplotyping the QTL region on chromosome 6. Based on allelic pattern of this QTL, 50 genotypes arranged in four haplotype groups (Table 6). Aghaeii seiah cultivar with high grain zinc content showed Dadras

haplotype in this QTL. Therefore, allele combinations 223-145 bp for markers RM8226 and RM7193, respectively, can be useful for grain zinc content breeding.

**Table 6.** Rice haplotypes produced by SSR markers located on QTL region associated with zinc content on chromosome 6 with reference to Dadras cultivar*.

| | | | | |
|---|---|---|---|---|
| RM8226 | ■ | ■ | | |
| RM7193 | ■ | | ■ | |
| Haplotype No | 1 | 2 | 3 | 4 |

1: Dadras*, Aghaeii seiah, 2: Rashti sard, Domsorkh, 111, Sadri, Khazar, 3: Bojar, Mehr, Domseiah, Hassan molaeii, Hassani, Haraz, Cheli, Line 29, Gil3, Line 27, Dorfak, Sepidrud, Fajr, Amol3, Onda, Norin22, Line 18, Line 13, Century patna, IR24, IR30, 4: Other cultivars.

**Discussion**

Rice provides energy to almost half of the world's population. Thus, increasing the zinc content of rice by traditional plant-breeding methods or molecular marker techniques has a great potential to mitigate wide spread zinc deficiency problem in humans (Ruel and Bouis, 1998; Bouis, 2000; Welch and Graham, 2004). Therefore, it is essential to understand genetic bases of accumulation of zinc. So far, several studies have reported the genetics of accumulation of zinc in the rice grains. Hanarida *et al.* (2002) evaluated 251 rice genotypes (local varieties, advanced lines, and improved varieties) and reported high variability for zinc (16.5-43 $mg.Kg^{-1}$) content in rice grains. Similarly, Anandan *et al.* (2011) evaluated 202 rice genotypes and reported that zinc concentration ranged from 3.00 to 38.60 $mg.kg^{-1}$ with a mean value of 15.84 $mg.kg^{-1}$. In present study, the average of zinc content (19.68 $mg.kg^{-1}$) 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 results, Anandan *et al.* (2011) and Gregorio *et al.* (2000) indicated that most of the traditional varieties contained high zinc and iron 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 zinc content was not part of the previous rice breeding program and the variability in improved varieties for zinc 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 zinc QTL on chromosome 8, haplotype of Mehr cultivar (contained high zinc) showed same SSR alleles with Dadras haplotype. Based on the result it is concluded, RM407, RM6925, RM22253 and RM22254 markers by allelic pattern of 166-184-327-266 bp are helpful allele combinations for identifying and utilizing zinc sources in breeding programs. Further, some genotypes such as Abjibuji, Hassan molaeii, Deilamani, Sangejo, had distinct haplotype patterns in this QTL, well-known zinc sources, suggesting that they could carry new zinc QTLs. Therefore, these cultivars could potentially be exploited to identify new QTLs of grain zinc in Iranian rice.

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. Also McCartney *et al.* (2004) identified 76 haplotypes among the 79 wheat lines for fusarium head blight resistance QTLs.

It is concluded that the genotypes belong to different haplotypes, such as Dadras and Neemat cultivars, can be used for hybridization to generate helpful recombinants in the segregating generations, the genetics and breeding programs for improvement of zinc content in rice grains.

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