WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 3, 1647-1672. Research Article ISSN 2277– 7105

IN SILICO STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF QTLS CONTROLLING GPC AND MICRONUTRIENT CONTENT AND IDENTIFICATION OF CANDIDATE GENE BASED QTLs SPECIFIC MARKERS

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Article Received on
19 Jan 2016,

Revised on 09 Feb 2016,
Accepted on 29 Feb 2016

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ABSTRACT

Rice (*Oryza sativa* L.) grain iron and zinc content is a polygenic complex trait having additive effect of multiple genes thus identification of QTLs and sequence analysis of genomic region encompassing them enable us to understand not only the inheritance of grain micronutrient content but also to develop Fe/Zn rich rice using marker assisted breeding techniques. Co-segregation analysis was performed with the F_6 mapping population derived from a cross between rice cultivar Swarna x Moroberekan. The grain Fe content ranged from 9.68 to 19.98 µg/g with an average of 16.87 µg/g and grain Zn content ranged from 15.85 to 20.84 µg/g with an average of 25.76µg/g among the 73 homozygous rice mapping population. Similarly the grain protein content ranged from 5.34% to 7.18% with an average of 9.23%. In order to identify novel SSR based molecular markers, 5 known QTLs (qFE-1, qFE-9, qZN-5, qZN-7 and qZN-11) identified[9] for grain Fe/Zn content in rice were analyzed using *in-silico* tools. Out of 1063 novel SSRs loci present within the 5 QTL regions, 161 Class I SSRs with 2-6 nt long repeat motifs and 12–80 nt repeat lengths were identified. On the basis of position of metal related transporter or membrane transporter genes primers were designed for 22

novel Class- I SSR and validated in the parents for their polymorphism. Out of 34 previously designed primers, 4 randomly selected RM markers and 22 novel SSR designed markers, only 18 markers were found to be polymorphic. The allelic segregation analysis indicated that *indica* parent Swarna contributed about 60.6% whereas the *japonica* parent Moroberekan contributed about 34.32% of total amplified alleles on an average which clearly showed departure from the theoretically expected 1:1 ratio of equal contribution from the two parents. Out of 18 polymorphic SSRs, the co-segregation analysis performed for the 5 randomly selected SSR's markers. The all five markers; gRMm7-2, gRMm7-3, gRMm33-2, gRMm34-1 and gRMm33-3 were significantly associated to grain iron and zinc contents.

KEYWORD: *in-silico*, Rice, allelic segregation, SSRs, Fe content, Fe/Zn, QTLs.

INTRODUCTION

Rice is vital staple food of more than half of the world's population, primarily the poor people living in Asia and Latin America. Approximately, 90 countries cultivate rice, farmers from irrigated upland, lowland and flood-prone areas across Asia are major rice producers. Rice represent single largest source of calories in the world.[6] The human body requires more than 22 minerals elements that can be supplied by an appropriate diet.[13] Rice has been a model plant for almost all genomics and molecular biology research owing to its small and compact genome. This research is important because the fruits of such research are going to affect major shift in food productivity and human nutrition.[12] Poor grain protein content in rice is an important cause of widespread protein malnutrition among rice eating population especially those residing in developing nations.[10] In India about 47% of children are suffering from protein energy malnutrition (PEM) with infants suffering more from clinical or sub clinical levels of protein deficiency.[16] However, rice is a poor source of essential micronutrients such as Fe and Zn.[1] Micronutrient malnutrition, and particularly Fe and Zn deficiencies (the so called 'hidden hunger'), affect over three billion people worldwide, mostly in developing countries.[18]

Enhancing GPC of rice is a recent food based approach that has gained attention not only of nutritionists and crop biologists but also of renowned economists all over the world.[4] Recently, a sustainable solution to mineral malnutrition termed as 'Biofortification' has been proposed of crop plants through enhanced in the edible portions of crop plants through agronomic intervention or genetic selection. Candidate gene approach is becoming a widespread method for characterizing Quantitative Trait Loci (QTLs) as well as Mendelian

traits in both the animal and plant systems. Candidate genes for economically important traits have been potentially useful in plant breeding. Apart from high amount of consumption the quality of protein of rice is considered as high because of better digestibility. To improve nutritive value of rice the preliminary step is to characterize genetic variability for grain protein content in germplasm and then to use this variability for breeding nutrient rich rice.[3] The complex polygenic traits are governed by Quantitative Trait Loci (QTLs) thus identification as well as characterization of QTLs controlling grain micronutrient contents in rice harbors great potential for Markers assisted selection (MAS) and QTLs introgression based breeding approaches to develop nutrient rich rice.

The candidate genes or DNA sequences with predicted functions serves as an important source to generate novel molecular markers within a given QTL region which is likely to show more stable association across the mapping populations or genetic stocks.[15] In this context the present study was undertaken with *in silico* structural and functional characterization of QTLs controlling GPC and micronutrient content and identification of candidate gene based QTLs specific markers, phenotypic characterization of parents and mapping population for grain micronutrient, protein and amino acid and genotyping of the mapping population for validation of novel molecular markers.

MATERIALS AND METHODS

The present investigation was carried out at Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur (CG).

Materials

The plant material used for this study includes the rice cross developed by Swarna x Morobreken and the F_6 population of 73 lines. Seeds of both parents and population were sown in pots under green house conditions for 2-3 weeks, in the Department of Plant Molecular Biology and Biotechnology.

Before analyzing the rice samples for total grain protein, iron and zinc content, 50gms of seeds of both parent and populations were subjected to dehusking by using polyurethane coated hand dehulling unit to avoid metal contamination.

Estimation of protein

Total protein content of brown rice grains of all samples were estimated by modified micro-Kjeldahl method[7] and the distilled samples were titrated against the 0.05 N Sulfamic acid until the first appearance of violate color as the end point. The titer value was used to calculate percent Nitrogen, which is then used to estimate total protein content by using conversion factor 5.95.[8]

Estimations of iron and zinc

Whole brown grains were subjected to di-acid mixture based digestion. Iron and zinc content was estimated by using standard method described under[5] guidelines using Atomic absorption spectrophotometer (AAS200).

Statistical analysis

The data obtained in present study was statistically analyzed using randomized block design, for checking genetic differences within these advanced breeding lines. The different parameters viz. standard deviation (SD), coefficient of variation (CV), coefficient of correlation, standard error (SE) was calculated.

Characterization of QTLs controlling grain Fe/Zn content in rice

Five QTLs namely qFE-1, qFE-9, qZN-5, qZN-7 and qZN-11 reported to govern high grain protein and FE/ZN content in rice by[9] were selected based on their higher phenotypic variance and lower LOD value. These four QTLs are located on chromosome 1, 5, 7, 9, and 11 of rice respectively (**Table 1**).

Table 1 List of QTLs present between the markers.

Sr. No.	QTL	Chromosome No.	Position on chromosome	Marker Interval	QTL length bp	BAC/PAC contig
1	qFE-1	1	41968722-42955596 bp	RG236 – C112	986874	8
2	qFE-9	9	14648372-17837010 bp	C472 – R2638	3188638	12
3	qZN-5	5	849198-1840480 bp	R3166 - RG360	991282	8
4	qZN-7	7	3033385-4415836 bp	RM234 – R1789	1382451	8
5	qZN-11	11	25472678-26529185 bp	RG118-C794	1056507	12

Identification of co-localized expressed sequence tags (ESTs)

The metal homeostasis related candidate gene sequences were analyzed for presence of known ESTs using PASA (Program to Assemble Spliced Alignments) program at TIGR rice genome browser (http://www.tigr.org/tdb/e2k/osa1/dnav/) and EST database at RGP website

(http:// rgp.dna. aafrc.go. jp/E/ publicdata /estmap2001/). The PASA program resulted in incorporation of high quality ESTs by transcript alignment in a genomic and full length cDNA (fl-cDNA). ESTs identified for each gene was then characterized for respective expression tissue library using digital northern and anatomy viewer search tools available in rice ESTs database. ESTs corresponding to a tissue library provided information about putative site of expression of the metal related genes in which it was identified.

Identification of Massive Parallel Signature Sequence (MPSS) tags

More than 20 MPSS libraries derived from diverse tissues root, leaves, stem, panicle and germinating seeds abiotically stressed (cold, drought and salt) tissues have been generated for *japonica* cultivar Nipponbare grown under different conditions as light and dark, different developmental stages and several biological replicates. The search resulted in 17 and 20 nt long MPSS tags, tag sequence, chromosome coordinate position, tissue library information and transcript abundance values such as TPM (transcripts per million) value.

Designing of primers

SSR primers were designed with selected putative SSR motifs using BatchPrimer-3 software. Primer designing was done by putting the nucleotide sequences in FASTA format with different parameters as default setting (**Table 2**).

Table 2 Specifications for primer designing.

Criteria	Optimum	Range
Length of target sequence to be amplified	250 bp	50-500bp
Tm	55^0C	45-60^0C
GC content	55%	50-60%
Length of primer	20bp	18-22bp

PCR analysis to detect parental polymorphism and validation of molecular marker

The 73 lines belonging to F_6 population derived from cross Swarna between and Moroberekan were used to obtain SSR genotypic data. Total rice genomic DNA was extracted from four-week old plants of the parental lines i.e. Swarna and Moroberekan as well as the cross population by the method described by by Dellaporta method (Dellaporta *et al.*, 1983) and for quantification, 3 µl of the DNA samples isolated from each line, along with standards of known quantity of DNA, was loaded on 0.8% agarose gel. After quantification, the final concentration of DNA was approximately 40 ηg/µl, for PCR analysis.

PCR analysis was done using the selected SSR and designed markers to identify the polymorphic loci between the parental lines, Swarna and Moroberekan and their F_6 population. Amplified products were resolved by electrophoresis on agarose gel along with 100 bp DNA ladder as molecular weight marker in 1X TAE buffer, and visualized with UV. Gels were photographed for parental polymorphism by using digital camera under photodyne of twenty two SSR primers. The primer exhibiting polymorphism on parents were further screened against population of 73 lines. Genotypic data were generated with a set of polymorphic primers. The banding pattern of population developed by each set of SSR primer was scored separately as Swarna like allele (A), Morobereken like allele (B), and Both alleles (H). QTL analysis was carried out in selected lines, single marker analysis was used to estimate association between marker and trait by using‘t’- test.

RESULTS AND DISCUSSION

In silico characterization of the QTL region governing Iron and Zinc content in rice

The characterization of QTLs related to mineral content in rice grains was carried out by the *in silico* approach. The QTLs selected for the study was previously identified by the.[9] Total five QTLs qFE-1, qFE-9, and qZN-5, qZN-7, qZN-11known for governing grain iron and zinc or QTLs AQTO33, AQTO34 protein content in rice were taken for the study. The details of the QTLs including chromosomal location, position and marker interval are presented in **Table 3**.

Table 3 Features of Quantitative Trait Loci known for governing Iron and Zinc content in rice.[9]

Sr. No.	QTL	Chromosome No.	Position on chromosome	Marker Interval	QTL length Bp	BAC/PAC contig
1	qFE-1	1	41968722-42955596 bp	RG236-C112	986874	8
2	qFE-9	9	14648372-17837010 bp	C472-R2638	3188638	12
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Two QTLs for Iron content in rice (qFE-1 and qFE-9), were found to be located on chromosome number 1 and 9 respectively, and three QTLs responsible for zinc content (qZN-5, qZN-7 and qZN-11), located on chromosome number 5, 7 and 11 respectively. Among all the five QTLs, qFE-9 was the largest and qFE-1 was the smallest QTL in terms of size (bp). Mining the genomic region of the target QTLs, revealed that all the five QTLs comprise various number of BAC/PAC clones. QTL qFE-1 and qFE-9 consist of 8 (3 BAC and 5 PAC

clones) and 12 BAC/PAC clones (6 BAC & 6 PAC clones) respectively, whereas QTLs qZN-5, qZN-7, and qZN-11 include 8 (1BAC & 7PAC clones), 8 (4BAC & 4PAC clones) and 12 BAC clones respectively.

Identification of putative candidate genes in the target QTL regions

In this study, genes were also analyzed for the presence of predicted number of exons, which revealed that gene major facilitator antiporter family underlying QTL qZN-7 has the highest number of predicted exons (17) whereas the minimum number of exon was found to be one and present underlying three genes encoding oxidoreductase/transition metal ion binding protein, cation efflux family protein and transporter, major facilitator family protein (**Table 4**).

Identification of co-localized Expressed Sequence Tags (ESTs) underlying putative candidate genes

In the present study analyzed distribution of identified ESTs in different tissues to predict putative site of expression of iron and zinc related putative candidate genes. Out of 9 iron and zinc related 5 QTLs genes analyzed *in silico*, ESTs were identified in 7 genes (metal cation transporter, oxidoreductase/ transition metal ion binding protein, 2Fe-2S iron-sulfur cluster binding domain containing, cation efflux family protein, heavy metal-associated domain containing protein, transporter, major facilitator family, ion channel nompc). A total of 113 EST_S were identified in 7 genes with maximum 64 ESTs in LOC_Os09g26650 2Fe-2S iron-sulfur cluster binding domain containing gene and minimum 5 ESTs in LOC_Os05g03780 gene encoding cation efflux family protein. The ESTs identified in each gene were then categorized according to their corresponding expression in tissue library such as flower, panicle, seed, leaves, roots, stem to understand putative site of expression. **Figure 1** shows total number of ESTs identified in each gene and their distribution in different tissue libraries. The ESTs corresponding to the gene encoding cation efflux family protein were found to express in callus, whole plant, shoot, flower, root callus, seed and leaf tissue libraries suggesting expression of the gene in these tissues. The ESTs identified in major facilitator family protein corresponded mostly to callus, flower, mixed, root and panicle tissues which suggested reproductive phase specific expression of these genes in rice. It was observed that ESTs overlapping cation efflux family protein also correlated to seed tissue suggesting role of this gene in grain uploading of micronutrients. One gene metal cation transporter carried

ESTs corresponding to root tissue which indicates that the above genes may participate in uptake and transport of metal ions within plants.

Similarly OsZIP9 gene express in floral tissue (panicle, stigma and ovary) and developing grains thus suggesting putative expression of OsZIP9 gene in reproductive plant parts. It was also observe that these genes did not express in tissue libraries including seedling, anther, stem, pistil and phloem etc showing their preferential expression in selected tissue types.

Characterization of putative candidate genes for grain iron and zinc content by MPSS signature analysis

A total of 35 MPSS tags (17 bp) were found corresponding to genes present in the QTLs controlling iron and zinc content. Out of 21 MPSS tags, nine signature tags belonged to class I (those present within the exonic region of the gene sequence) three belongs to II (within 500 bp potential 3_UTR) and class V (within intron, sense strand), one signature belonged class III while five signatures belonged to class IV (unannotated). No tags belonging VI (within intron, antisense strand) class of MPSS signature tags were identified. The abundance of a MPSS tag in a tissue library (root, leaf, stem, meristematic, ovary, pollen, stigma, panicle and developing seeds, germinating seedling) determined by its TPM (transcript per million) value is an indirect measure of level of corresponding gene expression. A TPM value of less than 5 corresponds to very low level while TPM value between 5-15 shows basal level of expression in Arabidopsis (Meyers *et al*., 2004), but only those MPSS tags having TPM $\geq$ 15 in atleast one tissue were included in the study. The MPSS analysis of gene encoding 2Fe-2S iron-sulfur cluster binding domain containing protein belonging to the QTL qFE-9 revealed very strong level of expression with the total TPM56388 value of and expressed in most of the tissue libraries such as young root, mature root, stem, mature leaves, germinating seeds, developing seed, meristematic tissue, roots, leaves, and under biotic and abiotic stresses, this gene was prominently expressed in *X. oryzae* and *M. grisea*, while moderate expression level was observed in ovary, pollen, mature.

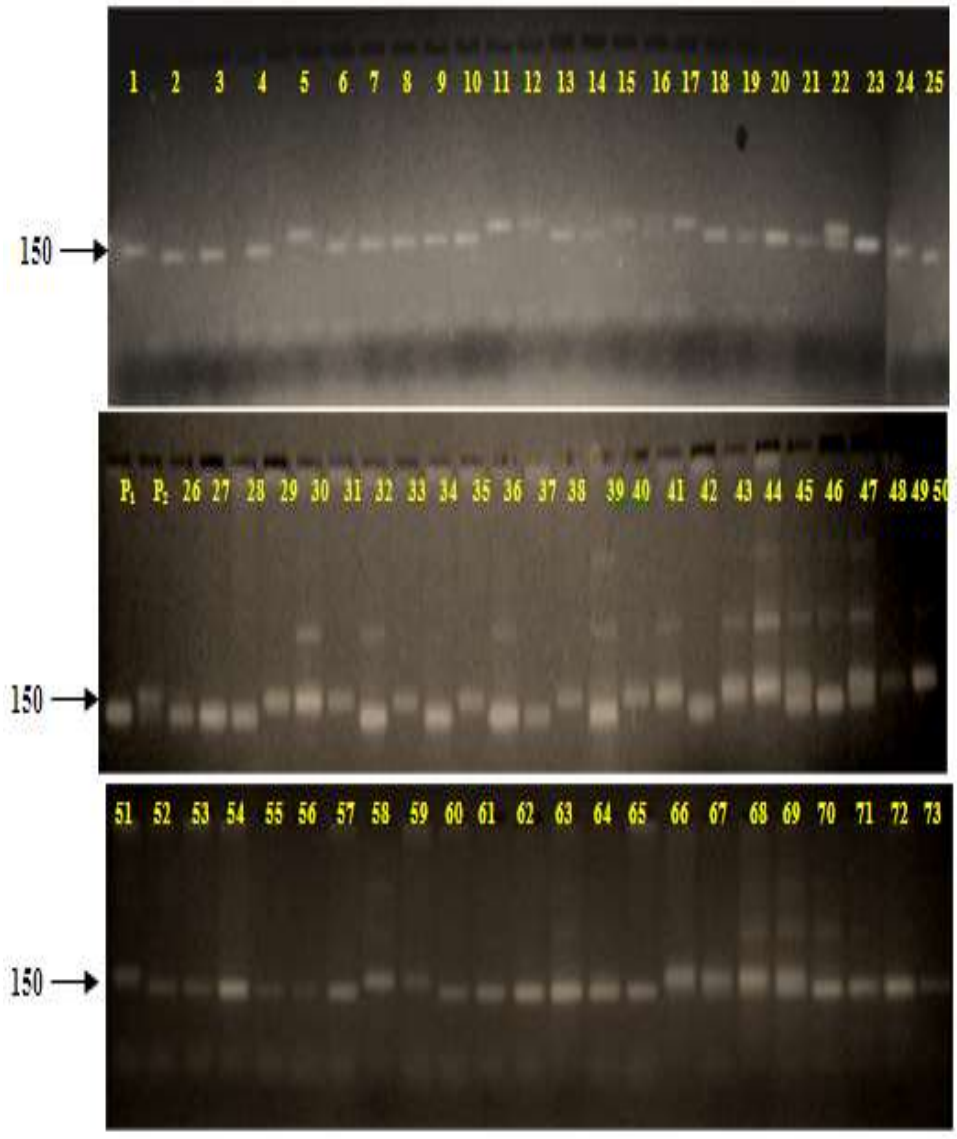


Figure 1 SSR profile of 71 rice lines derived from a cross between Swarna and Moroberekan using gRMm7-3 showing polymorphism (P1-Swarna, P2-Moroberekan; 1 to 73 mapping population).

Table 4 Detail of Putative candidate genes with their Clone ID, locus ID, GO ID, PFAM hits, chromosome position, no. of exons and no of predicted transmembrane domain for QTLs governing Iron and Zinc content in rice.

QTL	BAC/PAC Accession No.	Genes	Locus ID	Gene Ontology ID	PFAM hits	Chromosome Position	No. Of predicted exons	Predicted exons positions	Transmembrane domains
qFE-1	P0518C01	Metal cation transporter, putative	LOC_Os01g74110	GO: 0005215	PF02535	1	2	(66-731), (1301-1690)	9
qFE-9	OJ1123-B08	Oxidoreductase/ transition metal ion binding protein	LOC_Os09g27330	No Gene Ontology annotation found	No PFAM hits found.	9	1	(70 - 489)	3
	OJ1328-D07	2Fe-2S iron-sulfur cluster binding domain containing,putative	LOC_Os09g26650	GO:0003674	PF00111	9	7	(111-167), (693 -755), (1093-1158),(3530-3634), (3928 - 3975), (4049-4129), (4206-4295)	-
qZn-5	P0699E04	Cation efflux family protein, putative, expressed	LOC_Os05g03780	GO:0005215	PF01545	5	1	(1883 -3139)	6
qZN-7	P0524E08	Heavy metal-associated domain containing protein, expressed	LOC_Os07g43040	GO:0005215	PF00403	7	4	(121-396), (466-519), (1226 - 1378), (1763-1900)	-
	OJ1014-E09	Major facilitator antiporter family	LOC_Os07g08300	GO:0005215	PF07690	7	17	(40-267),(1487-1525), (2952-6359), (6504-7427), (8049-8108), (8249-8323), (8617-8670), (8760-8822), (8905-8952), (9061-9135), (9468-9668), (10219-10305), (10413-10496),	6

								(10578-10664), (11094-11138), (11537-11578), (12025-12027).	
qZN-11	OSJNBb009F15	Transporter, major facilitator family, putative, expressed	LOC_Os11g08370	GO:0005215	PF07690	11	1	(1117-2403)	12
	OSJNBb0084H09	Ion channel nompc, putative, expressed	LOC_Os11g07980	No Gene Ontology annotation found.	PF00023	11	4	(1837-2115), (2294-2614), (2902-2949), (4155-4277)	-

stigma and germinating seedling. Preferential expression of candidate genes was observed in the developing rice seeds indicating their role in the grain loading of micronutrients which is the most important aspect of the biofortification programs with the ultimate goal of enrichment of micronutrient in the edible plant part i.e. grain. The gene transporter, major facilitator family belonging to QTL qZN-11 showed the minimum level of expression with TPM value of 71. This gene is expressed in ovary and mature stigma, callus, under abiotic stress, leaves, and developing seeds. Rest six genes expressed at moderate level in the entire collection of tissue libraries. Higher expression level was observed for most of the genes under *X. oryzae* and *M. grisea* infected tissue libraries. This finding indicates the dynamic role of these genes in activities of plant defense and resistance related mechanisms. This finding is similar to the work of [19]. Computational expression profiling through EST and MPSS signatures revealed a considerable degree of commonalties in the two sequence tag based approaches. Collectively LOC_Os07g08300 (*qZN-7*), LOC_Os07g43040 (*qZN-7*), LOC_Os09g27330 (qFE-9) are the top ranking genes in all the datasets.

Grain micronutrient (Fe and Zn) contents in parents and mapping population

In this study grain iron and zinc content of F_6 mapping population developed from the cross between Swarna and Moroberekan was analyzed by using Atomic Absorption Spectrophotometer (AAS200) as per [5] protocol. Swarna is a popular *indica* rice cultivar having low grain Fe (8.63 µg/g) and Zn (14.38 µg/g) contents and Moroberekan is a *japonica* rice genotype having high grain Fe (13.63 µg/g) and Zn (21.38 µg/g). The results of elemental analysis revealed that the brown grain iron content ranged from 9.68 to 15.83 µg/g with an average of 16.87 µg/g (**Table 5**) and brown grain zinc content ranged from 15.5 to 22.7 µg/g with an average of 25.76 µg/g in parents and mapping population.

Table 5 Mean whole brown grain Iron and Zinc concentration in µg/g of 73 rice lines with parents Swarna and Moroberekan.

Sr. No.	Genotypes	Mean iron ± SEm µg/g	Mean zinc ±SEm µg/g	Sr. No.	Genotypes	Mean iron ± SEm µg/g	Mean zinc ±SEm µg/g
1	Swarna	8.63±0.06	14.38	40	SM 38	12.84±0.26	19.6±0.18
2	Moroberekan	13.63±0.53	21.38	41	SM 39	11.85±4.31	20.34±0.76
3	SM 1	13.42±0.55	20.42±0.80	42	SM 40	11.25±4.61	19.84±0.51
4	SM 2	13.25±3.61	18.76±0.62	43	SM 41	12.86±027	19.9±0.06
5	SM 3	9.85±1.24	16.68±1.67	44	SM 42	11.98±0.17	18.42±0.20
6	SM 4	9.84±5.31	18.31±0.85	45	SM 43	12.43±0.05	16.96±1.52
7	SM 5	11.05±4.73	17.66±1.17	46	SM 44	13.02±0.35	19.45±0.28
8	SM 6	13.60±0.64	21.28±0.62	47	SM 45	12.4±4.03	18.31±0.85
9	SM 7	12.93±0.30	20.96±1.07	48	SM 46	12.46±4.00	17.56±1.22
10	SM 8	12.42±0.05	19.68±0.17	49	SM 47	17.83±1.31	18.26±0.87
11	SM 9	13.26±0.47	17.73±1.14	50	SM 48	14.01±3.22	15.91±2.05
12	SM 10	12.74±0.21	19.21±0.40	51	SM49	15.13±2.66	20.9±0.43
13	SM 11	13.52±0.60	16.28±1.87	52	SM 50	14.43±3.01	21.24±1.21
14	SM 12	12.74±0.21	17.9±1.04	53	SM 51	13.83±3.31	17.7±1.16
15	SM 13	11.35±4.56	17.03±1.49	54	SM 52	12.9±3.78	20.84±1.01
16	SM 14	13.63±3.41	17.68±1.17	55	SM 53	13.5±3.48	20.33±0.15
17	SM 15	13.25±0.46	21.28±1.23	56	SM 54	14.68±2.89	17.53±1.24
18	SM16	13.23±3.61	16.53±1.74	57	SM 55	14.13±3.16	14.93±2.54
19	SM 17	13.61±0.64	15.63±2.19	58	SM 56	13.37±3.54	17.88±1.07
20	SM 18	12.48±0.08	19.41±0.30	59	SM 57	12.36±0.02	18.45±0.78
21	SM 19	13.52±0.60	19.5±0.26	60	SM 58	12.48±0.05	16.08±1.97
22	SM 20	12.68±3.89	21.01±0.49	61	SM 59	12.48±0.08	20.48±0.83
23	SM 21	15.83±2.31	20.8±0.38	62	SM 60	12.46±0.07	18.96±0.07
24	SM 22	13.18±3.64	17.61±1.20	63	SM 61	13.61±3.42	20.42±0.80
25	SM 23	11.96±4.25	20.42±0.80	64	SM 62	12.84±0.26	18.58±0.72
26	SM 24	10.28±5.09	19.42±0.30	65	SM 63	10.46±0.93	19.51±0.25
27	SM 25	13.41±3.52	16.25±1.88	66	SM 64	11.68±4.39	19.80±0.49
28	SM 26	10.06±5.20	16.56±1.72	67	SM 65	13.2±3.63	19.36±0.32
29	SM 27	12.7±3.88	16.15±1.93	68	SM 66	10.08±5.19	18.43±0.79

30	SM28	13.53±3.46	20.42±0.80	69	SM 67	12.51±3.97	18.24±0.29
31	SM 29	13.26±0.47	17.75±1.13	70	SM 68	12.82±0.25	17.96±0.43
32	SM 30	11.45±4.51	15.5±2.26	71	SM 69	12.32±0.00	18.16±0.92
33	SM 31	12.96±0.32	21.32±1.25	72	SM 70	11.68±0.32	20.42±0.80
34	SM 32	11.15±4.66	20.03±0.00	73	SM 71	12.42±0.05	19.45±0.28
35	SM 33	13.25±3.61	19.98±0.02	74	SM 72	12.42±0.05	20.23±0.10
36	SM 34	11.75±4.36	19.84±0.51	75	SM 73	9.68±5.39	20.42±0.80
37	SM 35	12.78±3.84	19.36±0.32				
38	SM 36	13.2±3.63	17.3±1.36				
39	SM 37	14.05±3.21	22.7±1.37				

Variance 1.16µg/g (iron) and 2.74µg/g (zinc); SEm 7.05µg/g (iron) and 0.5636u/g (zinc); CV –8.16% (iron) and 6.6 % (zinc).

Table 6 ANOVA for grain Fe content.

SV	D.F.	SS	MSS	F-cal	F-tab (5%)
Replication	2	1.8	0.9	.0.9	3.1
Treatment*	72	219.5	3.0	2.9	1.4
Error	144	1.0	1.0		
Total SS	**218**	**370**			

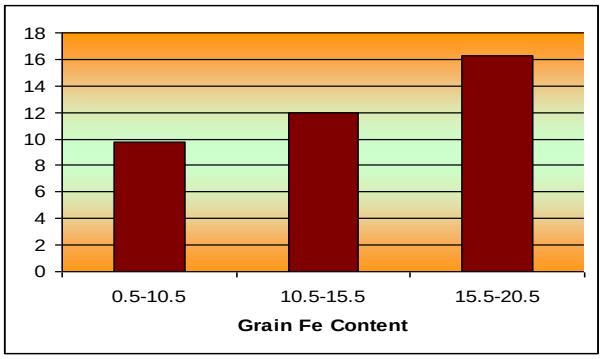
* Significant at 5% and 1% level of significance and 72 degrees of freedom.

Table 7 ANOVA for grain Zn content.

SV	D.F.	SS	MSS	F-cal	F-tab (5%)
Replication	2	2.2	1.1	1.2	3.1
Treatment*	72	628.4	8.7	9.2	1.4
Error	144	137.4	1.0		
Total SS	**218**	**74890.36**			

* Significant at 5% level of significance and 72 degrees of freedom.

The frequency of the number of plants falling in each class indicated normal distribution of the population for grain Fe and Zn trait (**Figure 2 and Figure 3**). The rice line SM 61 showed maximum grain Fe content (13.61 µg/g) which was more than Moroberekan and maximum grain Zn content 21.32 µg/g was observed in rice line SM 31 which is higher than Moroberekan.



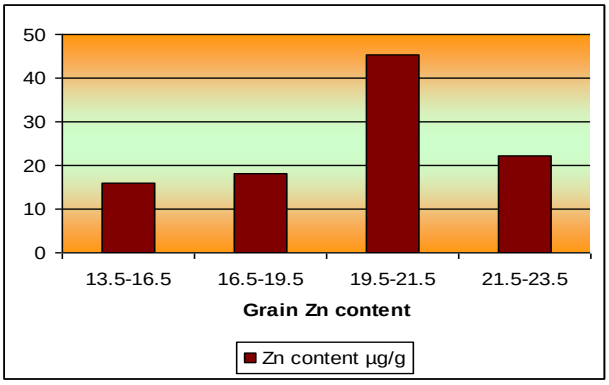


Figure 2 Frequency distribution of grain zinc content in cross population showing normal distribution

Figure 3 Frequency distribution of grain iron content in cross population showing normal distribution.

Grain protein concentration

Whereas significant differences in protein and amino acid content among Japanese native varieties were found, variety Brimful showed highest protein of 12.1% and lysine of 0.569g/100gm protein [15]. Wide variation for protein concentration in milled grains level from 2.8% to 9.9% of rice germplasm lines of Chhattisgarh have been reported by [3] (**Figure 4, Table 8 and 9**).

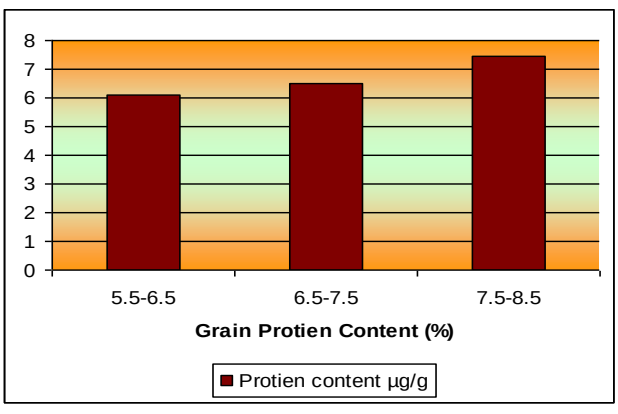


Figure 4 Frequency distribution of grain zinc content in cross.

Table 8 Mean whole brown grain protein.

Sr. No.	Genotypes	Mean Protien± SEm µg/g	Sr. No.	Genotypes	Mean Protien± SEm µg/g	Sr. No.	Genotypes	Mean Protien± SEm µg/g
1	Swarna	7.20±0.23	26	SM 24	6.75±0.00	51	SM49	5.43±0.02
2	Moroberekan	5.59±0.57	27	SM 25	7.07±0.17	52	SM 50	5.48±0.65
3	SM 1	7.06±0.16	28	SM 26	6.94±0.10	53	SM 51	6.89±0.63
4	SM 2	6.90±0.08	29	SM 27	7.18±0.22	54	SM 52	6.46±0.08
5	SM 3	7.01±0.14	30	SM28	7.00±0.13	55	SM 53	6.68±0.14
6	SM 4	7.10±0.18	31	SM 29	6.43±0.15	56	SM 54	7.18±0.03
7	SM 5	7.08±0.17	32	SM 30	6.84±0.05	57	SM 55	6.63±0.22
8	SM 6	6.87±0.07	33	SM 31	6.60±0.07	58	SM 56	6.65±0.01
9	SM 7	7.03±0.15	34	SM 32	7.10±0.18	59	SM 57	7.18±0.44
10	SM 8	7.11±0.19	35	SM 33	7.01±0.14	60	SM 58	6.60±0.21
11	SM 9	5.34±0.70	36	SM 34	6.44±0.15	61	SM 59	6.79±0.07
12	SM 10	6.91±0.09	37	SM 35	7.10±0.18	62	SM 60	6.80±0.02
13	SM 11	6.93±0.09	38	SM 36	7.11±0.19	63	SM 61	7.11±0.03
14	SM 12	6.50±0.12	39	SM 37	6.58±0.03	64	SM 62	7.20±0.19
15	SM 13	6.43±0.16	40	SM 38	6.79±0.03	65	SM 63	5.55±0.23
16	SM 14	7.15±0.21	41	SM 39	6.32±0.19	66	SM 64	6.47±0.59
17	SM 15	7.08±0.17	42	SM 40	6.73±0.21	67	SM 65	7.09±0.13
18	SM16	6.41±0.16	43	SM 41	7.32±0.00	68	SM 66	7.18±0.18
19	SM 17	6.72±0.01	44	SM 42	7.20±0.29	69	SM 67	6.73±0.22
20	SM 18	6.88±0.07	45	SM 43	6.44±0.23	70	SM 68	7.62±0.01
21	SM 19	6.57±0.08	46	SM 44	6.57±0.15	71	SM 69	6.32±0.44
22	SM 20	7.04±0.15	47	SM 45	6.80±0.01	72	SM 70	6.79±0.21
23	SM 21	6.79±0.03	48	SM 46	7.12±0.03	73	SM 71	6.71±0.02
24	SM 22	6.35±0.20	49	SM 47	5.41±0.19	74	SM 72	7.18±0.01
25	SM 23	6.65±0.04	50	SM 48	6.78±0.66	75	SM 73	7.06±0.12

Variance 7.7 SEm 3.72µg/g CV –7.79%.

Table 9 ANOVA for grain protein content.

SV	D.F.	SS	MSS	F-cal	F-tab (5%)
Replication	2	0.239	0.12	0.41	3.1
Treatment*	72	47.75	0.66	2.30	1.4
Error	144	41.57	0.29		
Total SS	218	89.57			

* Significant at 5% level of significance and 72 degrees of freedom.

Identification of novel SSR markers

Genomic region underlying five selected QTLs for grain micronutrient (iron and zinc) content were analyzed for identification of SSRs using Batchprimer3 SSR search tool (http://www.Batchprimer3). SSR markers were designed from the genomic DNA sequences.

A total of 1063 puatative SSRs were identified within genomic region encompassing the five QTLs including 152 loci in qFE-1, 482 loci in qFE-9,223 loci in qZn-5 and 157 loci in qZN-7. Occurrence of di and tri-nucleotide repeats occurred more commonly in all identified SSRs (**Table 10**). The tri-nucleotides repeats has been reported to be more common in eukaryotes especially cereals and legumes plant genomes. The potential of putative SSR to be used as a marker depends on repeat motif, number of repeats and position in gene.

Out of 1063 putative SSRs, 141 class I SSRs (more than 20 nt long) were selected such that they covered each 100-200 Kb fragment of the QTLs in each question. Out of the total Class I SSRs, 22 putative SSRs were found to be present in immediate vicinity of a metal transporter or cross membrane transporter family protein or hypothetical protein encoding genes (**Table 11**) and were used to design ~ 22 nt primers using BatchPrimer-3 software. The newly designed primers from 22 novel SSRs loci were used to fine map the genomic region encompassing five known QTLs controlling grain iron and zinc content.

Table 10 Total 1063 putative SSRs identified within five known QTLs controlling grain iron and zinc content in rice.

QTLs	Putative SSRs	Motifs
qFE-1 for grain iron content in rice	**152**	(acg)7, (tgg)6, (cag)6, (tcta)6, (gcc)7, (gtg)6, (ct)9, (tac)6, (ctgc)5, (gt)9, (ac)9, (gtt)6, (ta)26, (ta)40, (at)10, (g)23, (cc)9, (at)19, (at)33, (ta)16, (ctagct)3, (tc)9, (ctctc)4, (gaa)7,(tga)7, (ag)9, , (at)10, (gaa)6, (catca)4, (aat)7, (ccg)6, (cgc)6, (cgctcc)3, (cgc)6, (gga)6, (gga)6, (at)10, (tca)6, (aat)8, (tcc)8, (ag)10, (ga)14, (cct)6, (g)20, (cgc)9, (gca)7, (gcc)6, (tcc)8, (ta)32, (cgg)6, (c) 25, (cgc)6, (ct)13, , (gcc)6, (ta)41, (a)23, (gcc)7, (cgt)9, (gac)8, (gcc)7, (cgagct)4, (gcg)6, (gca)6, (gcg)6, (gcg)8, (tc)14, (ga)15, (tca)7, (acg)6, (c)22, (at)14, (at)11, (ta)14, (ct)9, (ta)23, (tatg)20, (cgc)6, (acat)6, (at)19, (cagctc)3, (ta)14, (aat)26, (atta)5, (ataac)5, (attagc)3, (aag)10, (ctc)7, (tttttc)3, (ttctc)4, (aatca)4, (tc)9, (ggt)6, (ggt)6, (tt)11, (at)25, (at)20, (aaag)5, (gat)7, (ctg)6, (ga)9, (gaa)6, (cttt)5, (aag)6, (aag)14, (gt)20, (ta)13,(tatg)6, (ta)10, (ta)15, (gac)7
qFE-9 for grain iron content in rice	**482**	(ag)9, (ata)6, (tctt)6, (gt)9, (cgc)7, (gag)8, (tc)9, (tga)6, (cgg) 6, (tgga)5, (aaacaa)3, (ag)15, (gcgaga)3, (ct)17, (cgccgt)3, (cgccgt)3, (at)42, (tg)16, (tct)6, (ggc)6, (ttc)22, (ttaa)6, (ag)10, (at)13, (ag)15, (cag)6, (aat)10, (gga)8, (ggc)6, (ccg)6, (cgg)6, (gcaggt)3, (ta)10, (ga)14, (cga)6, (cga)6, (cga)6, (cacctc)4, (aat)6, (ccac)5, (tg)15, (ta)24, (tatg)18, (ctgcga)3, (cgc)6, (tc)10, (ga)11, (aata)6, (ggggcg)3, (gga)8, (ggacaa)3, (gcg)6, (ttc)6, (agc)7, (cgggtg)3, (ct)10, (gcg)10, (gccgag)3, (gcg)6, (at)13, (gcc)6, (gtc)6, (gag)6, (ctg)6, (ac)10, (tagct)4, (ccg)6, (ggat)6, tctttc)3, (ccg)6, (at)14, (c)21, (gcct)5, (cca)6, (c)22, (ct)13,

		(tatc)11, (aataaa)3, (ctggt)4, (cgg)6, (gct)7, (taatag)3, (at)26, (ccg)6, (cct)7, (gcg)6, (gg)9, (ggt)7, (ta)13, (ccg)7, (ggc)6, (taga)7, (gcgt)5, (gcg)7, (t)20, (ta)13, (taat)5, (aattca)3, (gccgga)3, (ct)9, (ta)26, (at)42, (ct)30, (ta)31, (tg)20, (gct)9, (ccg)8, (ct)12, (ct)12, (ta)30, (ta)10, (ag)9, (gcg)6
qZN-7 for grain zinc content	**157**	(ct)13, (tgg)6, (ctcct)4, (cgg)7, (gag)9, , (gcc)6, (ctt)12, (ag)22, (gct)6, (ttctt)4, (cgg)6, (ctt)6, (ag)17, (ag)20, (tc)10, (cag)8, (cgg)6, (c)21, (gg)9, (cgc)6, (tatc)6, (tatc)9, (ta)16, (gtg)6, (cg)9, (ct)11, (at)47, (ta)10, (at)18, (ac)9, (tc)9, (ct)10, (cgt)7, (gcg)7, (ta)38, (tc)19, (cttt)5, (cata)7, (taat)7, (cgg)6, (gga)6, (ga)11, (tc)16, (tgc)6, (ggc)6, (ag)12, (aga)6, , (ta)18, (gg)9, (ggc)7, (ag)17, (at)18, (gag)6, (tttc)6, (cgg)6, , (ta)38, (ta)40, (ta)11, (atg)7, (tgg)8, (ct)15, (cgg)6, (cgg)6, (ggc)7, (cgg)8, (tc)10, (gcc)7, (gcc)6, (ta)23, (ta)22, (gatc)5, (ct)20, (tc)10, (ttggt)4, (cgc)6, (gcg)8, (gcg)8, (gcg)6, (ttc)7, (ggc)6, (ag)10, (tt)9, (ttc)6, (ttaa)6, (ct)12, (cca)7, (ggc)6, (ta)9, (at)10, (aat)24, (ta)24, (cgc)9, (at)9, (atta)5, (ttc)7, (at)16, (ac)10, (at)10, (tc)18, (gg)9, (ag)14, (ag)15, (at)10, (at)11, (at)13
qZN-5 for grain zinc content	**110**	(ac)10, (gtt)8, (ta)23, (ta)11, (at)20, (g)23, (cc)9, (at)15, (at)23, (ta)26, (ctagct)3, (tc)9, (ctctc)4, (gaa)7,(tga)7, (ag)9, , (at)10, (gaa)6, (catca)4, (aat)7, (ccg)6, (cgc)6, (cgctcc)3, (cgc)6, (gga)6, (gga)6, (at)10, (tca)6, (aat)8, (tcc)8, (ag)10, (ga)14, (cct)6, (g)20, (cgc)9, (gca)7, (gcc)6, (tcc)8, (ta)32, (cgg)6, (ac) 25, (cgc)6, (ct)13, , (gcc)6, (ta)41, (a)23, (gcc)7, (tca)7, (acg)6, (at)22, (at)14, (at)11, (ta)14, (ct)9, (ta)23, (tatg)20, (cgc)6, (acat)6, (at)19, (cagctc)3, (ta)14, (cga)6, (cga)6, (cacctc)4, (aat)6, (ccac)5, (tg)15, (ta)24, (tatg)18, (ctgcga)3, (cgc)6, (tc)10, (ga)11, (aata)6, (gggcg)3, (aga)8, (gcaa)3,
qZN-11 for grain zinc content	**163**	(ga)15, (cga)7, (cga)8, (cga)9, (cacctc)7, (aat)3, (ccac)25, (tg)25, (ta)24, (tatg)15, (ctgcga)32, (cgc)16, (tc)20, (ga)11, (aata)6, (ggggcg)3, (gga)18, (ggacaa)3, (gcg)6, (ttc)6, (agc)7, (cgggtg)3, (ct)10, (gcg)10, (gccgag)3, (gcg)16, (at)13, (gcc)6, (gtc)6, (gag)6, (ctg)16, (ac)10, (tagct)4, (ccg)16, (ggat)26, tctttc)3, (ccg)6, (at)14, (c)21, (gcct)5, (cca)6, (c)22, (ct)13, (tatc)11, (aataaa)3, (ctggt)4, (cgg)16, (gct)7, (taatag)3, (at)26, (ccg)6, (cct)7, (gcg)6, (gg)9, (ggt)27, (ta)13, (ccg)7, (ggc)6, (taga)7, (gcgt)5, (gcg)7, (t)20, (ta)13, (taat)5, (aattca)3, (gccgga)3, (ct)9, (ta)26, (at)32, (ct)20, (ta)21, (tg)20, (gct)4, (ccg)9(cgt)9, (gac)8, (gcc)7, (cgagct)4, (gcg)6, (gca)6, (gcg)6, (gcg)8, (tc)14, (ga)15,

Table 11 Genes present in the region encompassing 22 selected class -1 SSR loci.

QTL	Sr. No.	Primer	Encompassing gene	Function
qFE-1	1	gRMm1-1	LOC_Os01g73590	Transporter family protein, putative
	2	gRMm1-2	LOC_Os01g74110	Metal cation transporter, putative
qFE-9	3	gRMm9-1	LOC_Os09g28560	Protein phosphotase protein, putative

	4	gRMm9-2	LOC_Os09g26460	Protein binding protein, putative
	5	gRMm9-3	LOC_Os09g27330	Oxidoreductase/ transition metal ion binding protein
	6	gRMm9-4	LOC_Os09g26650	2Fe-2S iron-sulfur cluster binding domain containing, putative
	7	gRMm9-5	LOC_Os09g26900	ctr copper transporter family protein, putative,
	8	gRMm9-6	LOC_Os09g27580	Potassium transporter, putative,
	9	gRMm9-7	LOC_Os09g28610	Protein transport protein, putative,
	10	gRMm9-8	LOC_Os09g26290	Amino acid transporter family protein,
	11	gRMm9-9	LOC_Os09g28160	Phosphate carrier protein, mitochondrial precursor, putative,
	12	gRMm9-10	LOC_Os09g27960	Transmembrane protein 50A, putative,
	13	gRMm9-11	LOC_Os09g29430	Citrate transporter, putative,
	14	gRMm9-12	LOC_Os09g24980	Vesicle transport v-SNARE protein, putative,
qZn-5	15	gRMm5-1	LOC_Os05g03000	Ion channel nompc, putative,
qZN-7	16	gRMm7-1	LOC_Os07g43040	LTPL56 - Protease inhibitor/seed storage/LTP family protein precursor, expressed
	17	gRMm7-2	LOC_Os07g43040	Heavy metal-associated domain containing protein, expressed
qZN-11	18	gRMm11-1	LOC_Os11g0760	ABC-2 type transporter domain containing protein, expressed
	19	gRMm11-2	LOC_Os11g06820	Transmembrane amino acid transporter protein, putative, expressed
	20	gRMm11-3	LOC_Os11g06410	Homeodomain, putative, expressed
	21	gRMm11-4	LOC_Os11g08370	Transporter, major facilitator family, putative, expressed
	22	gRMm11-5	LOC_Os11g07980	Ion channel nompc, putative, expressed

Validation of identified SSRs markers

Parental polymorphism analysis using SSR primers

In this study cross validated 34 previously designed primers and 4 known random rice microsatelite (RM SSRs) markers and tested them in Swarna X Moroberekan F_6 population

to detect any polymorphism between the parents Swarna and Moroberekan. Out of a total of 38 markers screened, 18 markers showed polymorphism between the two parents Swarna and Moroberekan (**Figure 5**). The monomorphic or polymorphic amplification of the 34 new SSRs generated based on the 5 QTLs for grain iron and zinc content in rice have validated them as new generation genomic DNA based markers. This finding shows a higher level of polymorphism/ differences in the sequences of the *indica* and *japonica* subspecies of rice in the QTL region under study. Out of 18 polymorphic primers, 5 primers were selected randomly and were used for co segregation studies in the mapping population.

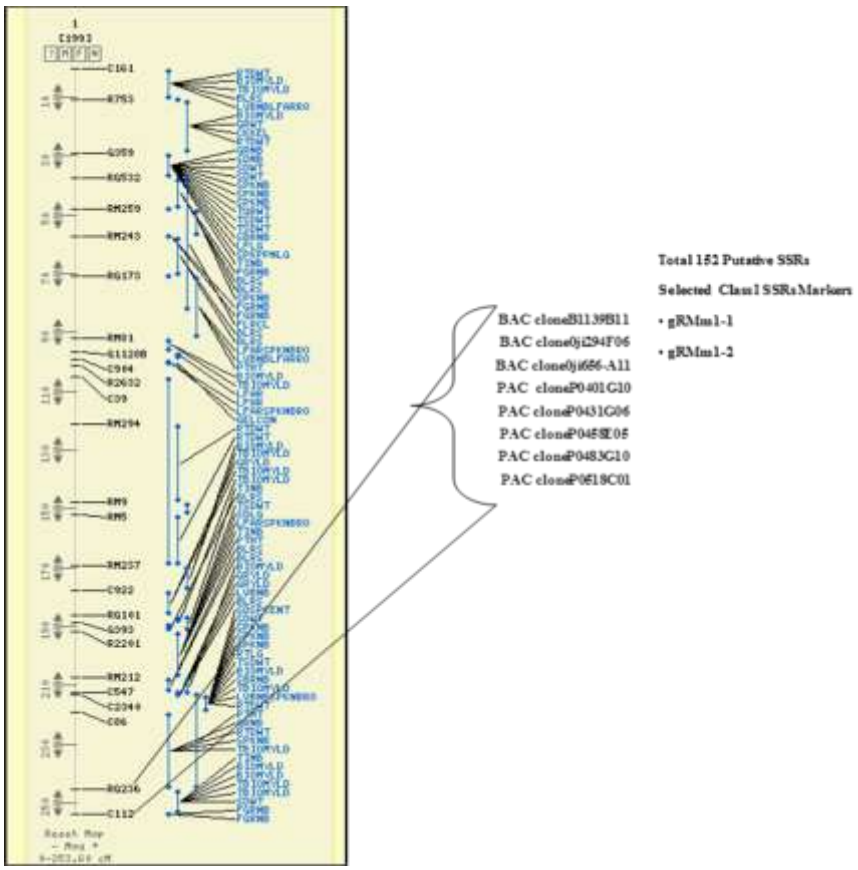


Figure 5 Map position of QTL qFE-1 on Ch # 1 along with co-localized putative SSRs markers identified.

SSR based genotyping of mapping population

The five novel SSRs showing polymorphism with parents were selected for genotyping of the mapping population. The genotypic data thus generated (**Figure 6**) was analyzed for segregation of Swarna and Moroberekan like alleles in the population. The Scoring of bands was done by designating the Swarna parent as A allele, Moroberekan parent as B allele. A perusal of gel pictures/genotyping data indicated amplification of either Swarna like or

Moroberekan like allele in the breeding lines, while some of the lines showed both Swarna and Moroberekan like alleles and thus were considered as heterozygous. It was found that the Swarna contributed about 50.1% of its trait (on the mean basis) whereas the Moroberekan contributed about 44.93% of its trait on similar basis. The variation in trait was represented departure from the theoretically expected ratio of 1:1 i.e. equal contribution from both the parents. The rice lines showing Swarna like allele were found to carry low Fe and Zn content while those showing Moroberekan like allele showed comparatively higher grain Fe/Zn content. Yet many rice lines of mapping population having Moroberekan like allele were found to contain lesser grain micronutrient contents. All the 5 polymorphic novel SSRs markers show a significant deviation from the expected 1:1 ratio.

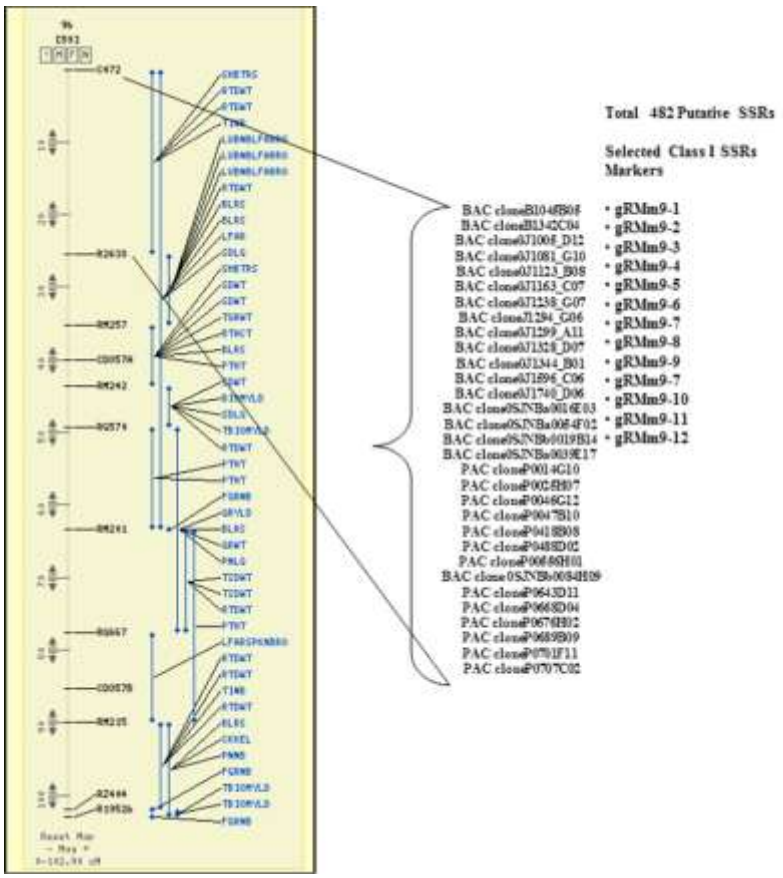


Figure 6 Map position of QTL q FE-9 on Ch #9 along with co-localized putative SSRs markers identified.

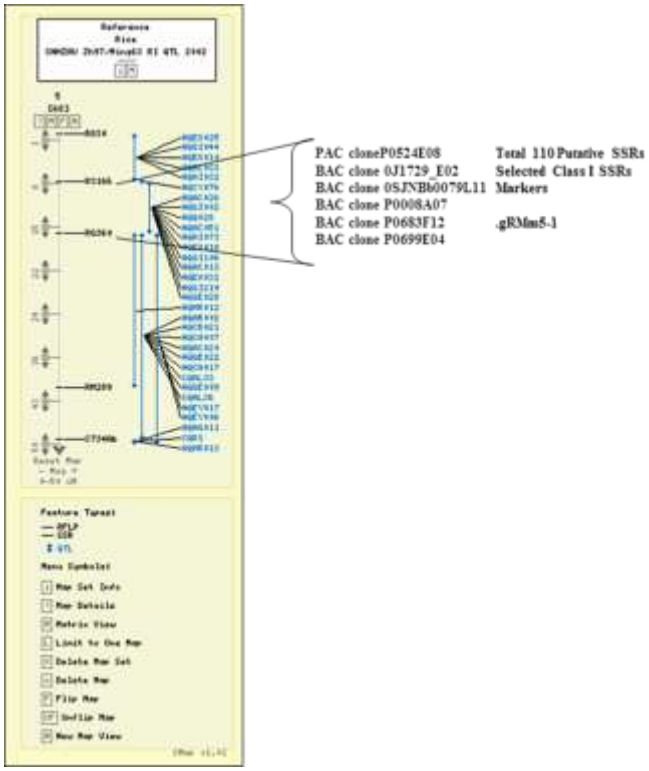


Figure 7 Map position of QTL qZN-5 on Ch # 5 along with co-localized putative SSRs markers identified.

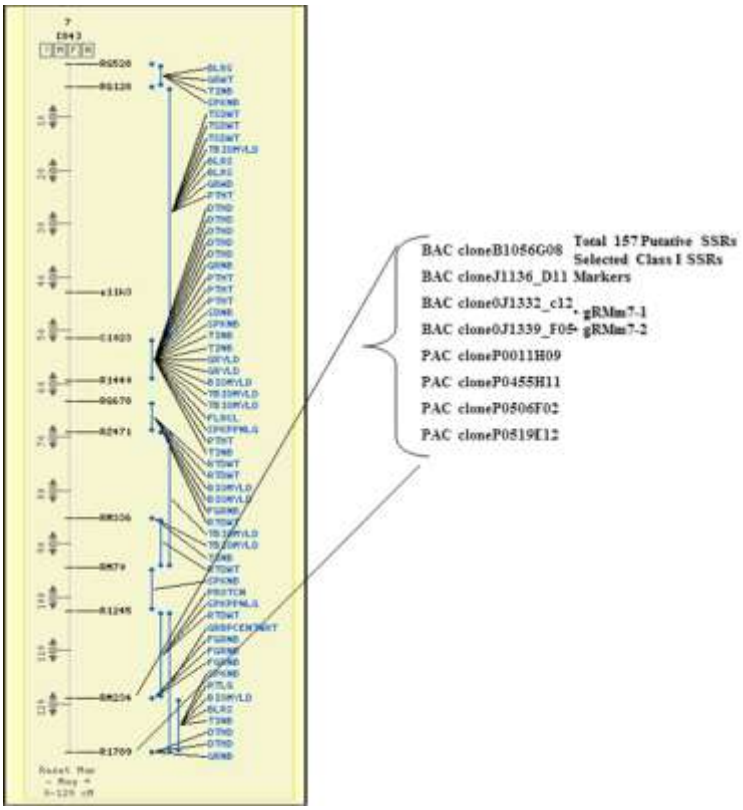


Figure 8 Map position of QTL qZN-7 on Ch # 7 along with co-localized putative SSRs markers identified.

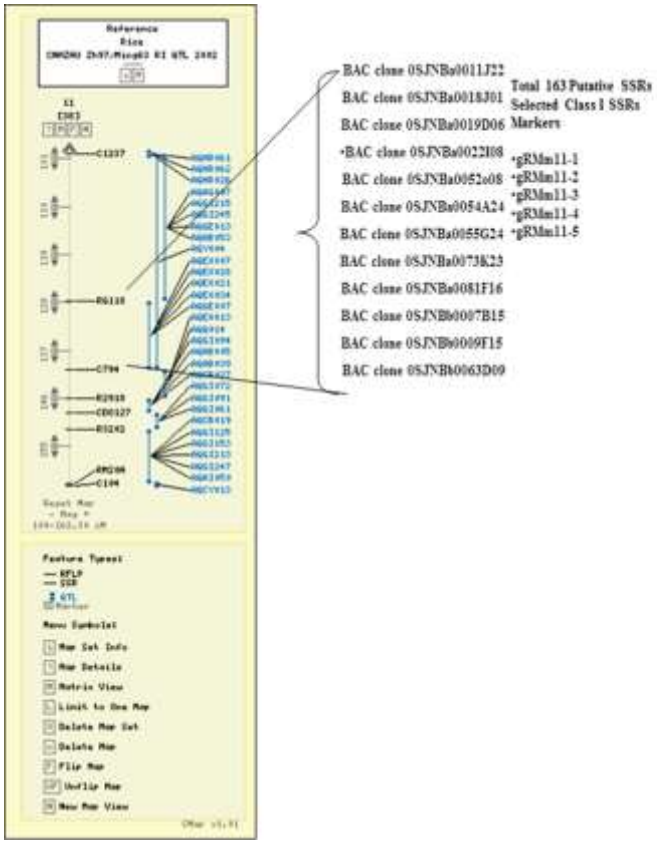


Figure 9 Map position of QTL qZN-11 on Ch # 11 along with co-localized putative SSRs markers identified.

Association mapping

Single marker association mapping technique was used to identify the association of SSRs markers to iron, zinc contents in brown rice grains. 't' value was determined for each of the polymorphic primer to analyze its significant association to grain micronutrient content which is presented in the **Figure 10**, **Table 15** was checked with 't' value at 72 degree of freedom at 5% level of significance. The analysis revealed that the polymorphic marker gRMm33-3 was significantly associated to grain Iron and Zinc contents. Similarly 4 markers namely gRMm7-2, gRMm7-3, gRMm33-2 and gRMm34-1 showed significant associations with grain Iron and Zinc content. Determination of association of a marker with a trait is the basic principal of association mapping. The mapping population is partitioned into different phenotypic classes based on the variability for the trait. The correlative statistical analysis of the genotypic data of the marker locus for individual genotype is performed with the phenotypic classes and forms the basis of association mapping. The failure of independent segregation of marker loci with the phenotypic class is said to display "linkage disequilibrium"[14] and QTLs identification is based on linkage disequilibrium. Several

statistical methods and softwares have been developed to determine association of marker loci with a trait including Single Marker Analysis, interval mapping, MAPMAKER, QTL mapper and Q Gene *etc.*[21, 20]

Table 15 ‘t’- test of the polymorphic primers for Zn and Fe content.

Sr. No.	Primer	Chromosome	t-value (Zinc)	t-value (Iron)	Association with grain Fe content	Association with grain Zn content
1	gRMm7-3	7	851.16	1343	Associated	Associated
2	gRMm34-1	3	463.12	661	Associated	Associated
3	gRMm33-2	2	956.52	1449	Associated	Associated
4	gRMm33-3	2	1074.66	1630	Associated	Associated
5	gRMm7-2	7	492.15	690	Associated	Associated

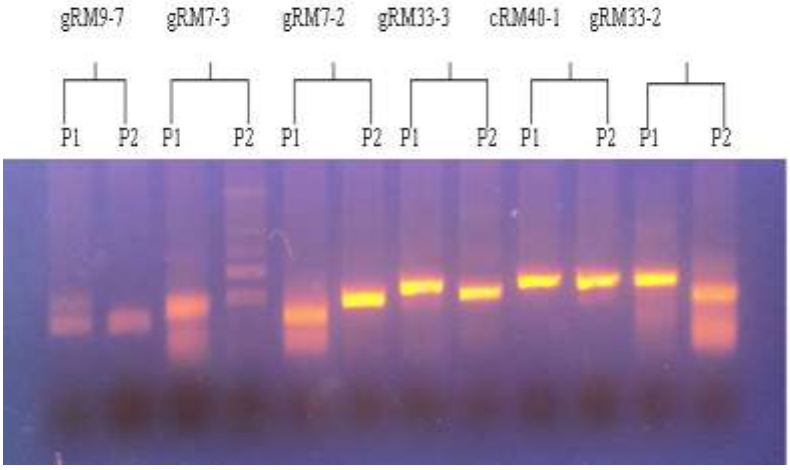


Figure 10 Parental polymorphism profile using QTL specific SSR marker M- marker, P1-Swarna, P2- Moroberkan.

CONCLUSIONS

- The grain protein content of different rice lines of population derived from cross between Swarna and Moroberekan ranged from 5.34 to 7.18% with an average of 9.23%.
- The brown grain Iron content ranged from 9.68 to 19.98 µg/g with an average of 16.87 µg/g while, the brown grain Zinc content was found to range from 15.5 to 20.84 µg/g with an average of 25.76 µg/g, in mapping population. The coefficient of variation (CV) for grain Fe and Zn content was found to be 8.16 % and 6.6 %, respectively. The analysis of variance using Randomized Block Design (RBD) indicated significant variation in grain Fe, Zn and protein content among 73 rice lines belonging to Swarna X Moroberekan mapping population at1% and 5% level of significance.

- A total of 1063 SSRs have been identified in the genomic region of 5 known QTLs and twenty two novel SSR primers have been designed from the selected Class I SSR loci which are needed to be experimentally validated in the mapping population.
- Out of a total of 38 previously designed primers, 18 have shown polymorphism between the parents Swarna and Moroberekan. This indicates a higher level of sequence differences and polymorphism between the *indica* and *japonica* subspecies of rice in the QTL regions under study.
- The co-segregation analysis for phenotypic and SSR genotypic data generated from the F_6 population revealed that 60% were Swarna like alleles and 35% were Moroberekan like alleles.

The association analysis between the markers and trait revealed that three novel QTL specific SSRs markers namely gRMm7-2, gRM 7-3 gRMm33-2 and gRMm34-1 were associated to grain iron content and zinc. Significant association was found between the novel SSRs markers and grain Fe and Zn contents in rice for the QTL analyzed in the study.

ACKNOWLEDGEMENT

Authors are grateful to Department of Biotechnology, Govt. of India for proving the funds and the work was done as an post graduate programme, so the authors are especially grateful to the Professor and Head of Department, I.G.K.V.V., Raipur (CG) for representing their help and support in pursuing this work.

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