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Mapping and characterization of putative candidate genes for grain iron and zinc content in rice by MPSS signature analysis

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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.

Keywords: *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. 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

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 greenhouse 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, iron and zinc

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 Sulfuric acid until the first appearance of violate color as the end point. The titer value was used to calculate % nitrogen, which is then used to estimate total protein content by using conversion factor 5.95 [8].

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.

Identification of co-localized (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.

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

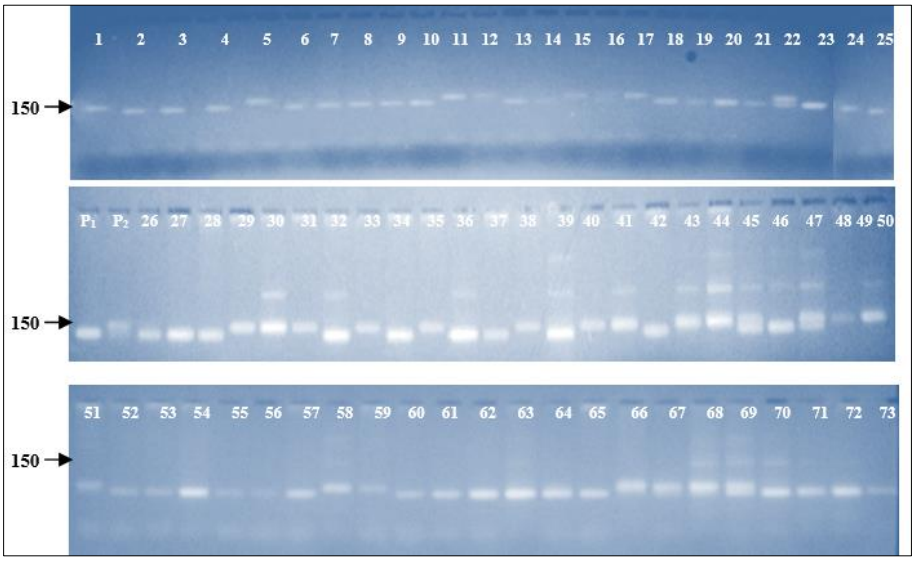

Fig 1: SSR profile of 71 rice lines derived from a cross between Swarna and Moroberekan using gRMm7-3 showing polymorphism (P1-Swarna, P2-Moroberekan; 1 to 73 mapping population

Table 1: Detail of Putative candidate genes with their Clone ID, locus ID, GO ID, PFAM hits, chromosome position, no. of exons and no of predicted trans membrane 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), (10578-10664), (11094-11138), (11537-11578), (12025-12027).	6
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)	-

This finding indicates the dynamic role of these genes in activities of plant defense and resistance related mechanisms. 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

Table 2: 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	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)			
38	SM 36	13.2±3.63	17.3±1.36				
39	SM 37	14.05±3.21	22.7±1.37				

Table 3: 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 4: 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

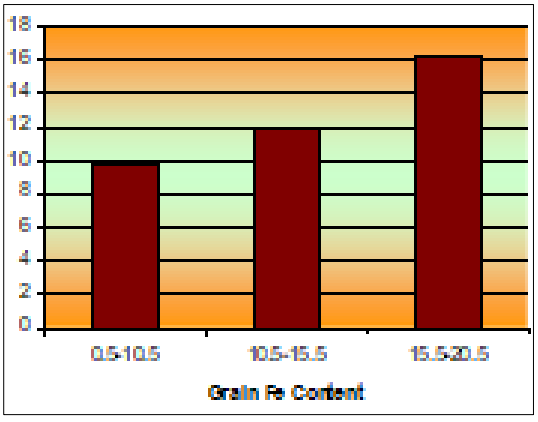

Fig 2: Frequency distribution of grain zinc content in cross population showing normal distribution.

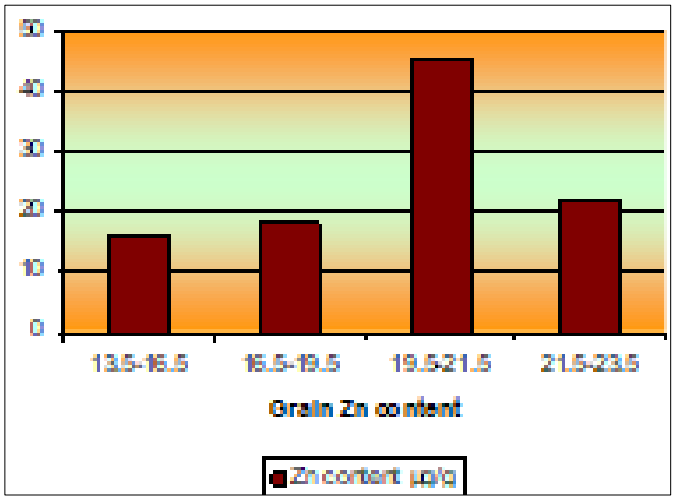

Fig 3: Frequency distribution of grain iron content in cross population showing normal distribution

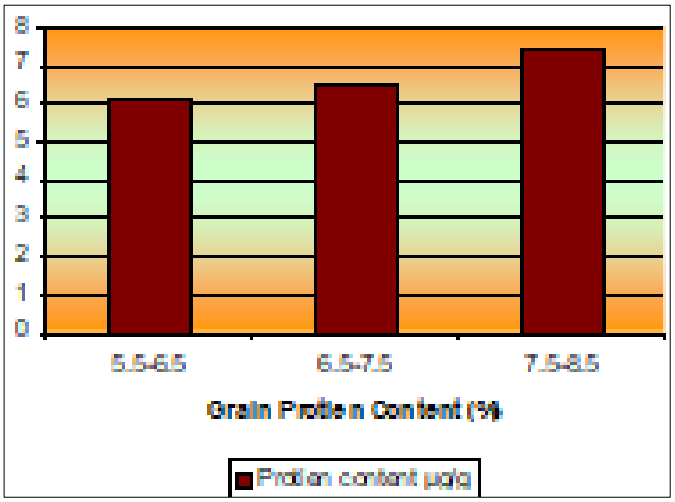

Fig 4: Frequency distribution of grain zinc content in cross population showing normal distribution

Grain protein concentration

Table 5: 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 6: 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

Table 7: 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	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,

content in rice		(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 8: 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 and Parental polymorphism analysis using SSR primers

In this study cross validated 34 previously designed primers and 4 known random rice microsatellite (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). Out of 18 polymorphic primers, 5 primers were selected randomly and were used for co segregation studies in the mapping population.

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 was analyzed for segregation of Swarna and Moroberekan like alleles in the population (Figure 6). 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. 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.

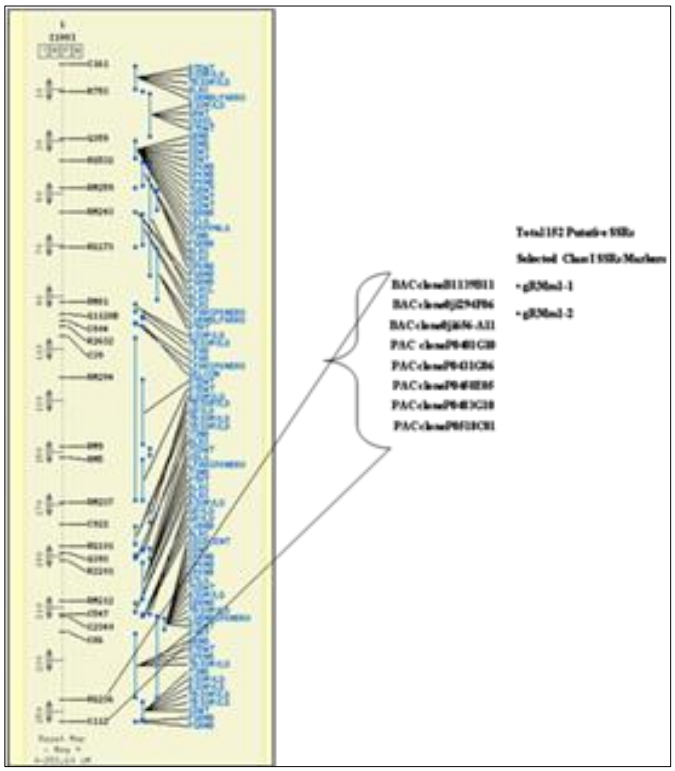

Fig 5: Map position of QTL qFE-1 on Ch # 1 along with co-localized putative SSRs markers identified

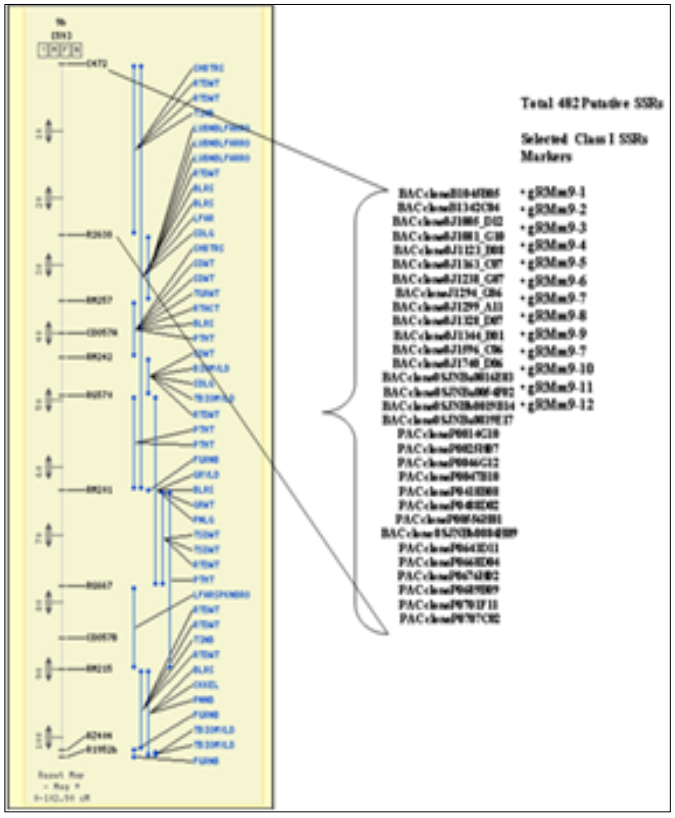

Fig 6: Map position of QTL q FE-9 on Ch #9 along with co-localized putative SSRs markers identified

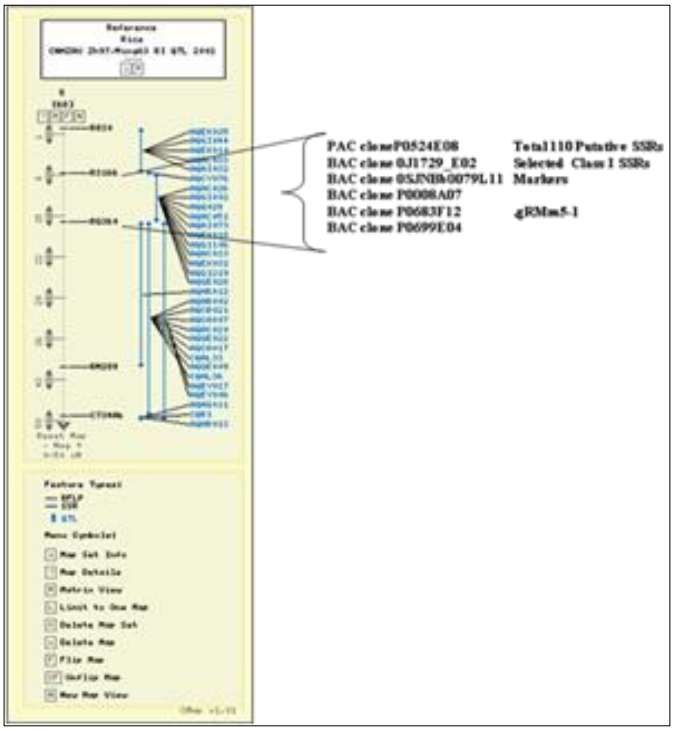

Fig 7: Map position of QTL qZN-5 on Ch # 5 along with co-localized putative SSRs markers identified

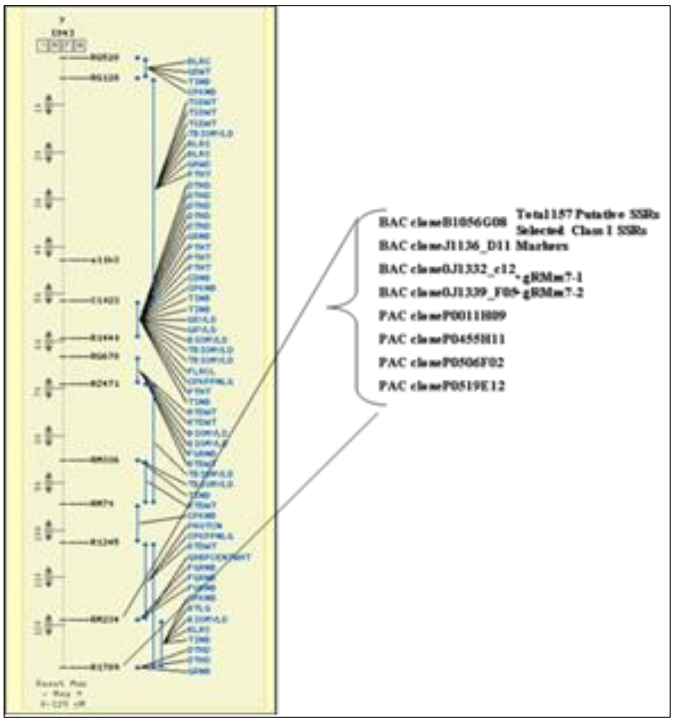

Fig 8: Map position of QTL qZN-7 on Ch # 7 along with co-localized putative SSRs markers identified

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 9 was checked with 't' value at 72 degree of freedom at 5% level of significance. The failure of independent segregation of marker loci with the phenotypic class is said to display "linkage disequilibrium" [14].

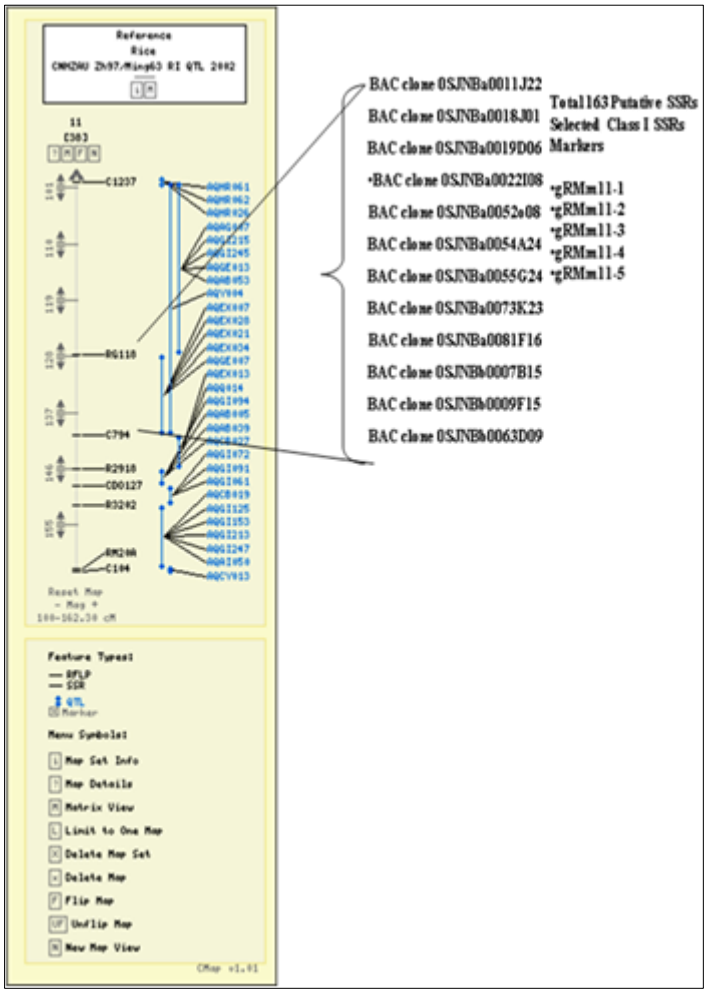

Fig 9: Map position of QTL qZN-11 on Ch # 11 along with co-localized putative SSRs markers identified

Table 9: '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

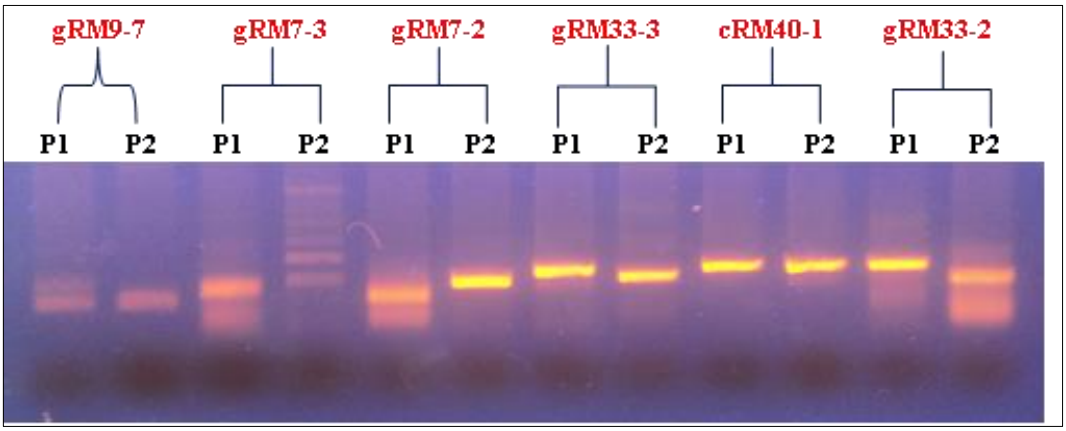

Fig 10: Parental polymorphism profile using QTL specific SSR marker M- marker, P1-Swarna, P2- Moroberekan

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 and zinc content ranged from 9.68 to 19.98 µg/g and 15.5 to 20.84 µg/g with a significant average of 16.87 µg/g and 25.76 µg/g respectively.

- 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.
- 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 three novel QTL specific SSRs markers namely gRMm7-2, gRM 7-3 gRMm33-2 and gRMm34-1 were significantly associated to grain iron content and zinc.

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