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Molecular Characterization of Rice (*Oryza sativa* L.) Genotypes Using Trait Specific Markers for Grain Zinc Content

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ABSTRACT

Micronutrient malnutrition is a serious health problem worldwide, affecting more than three billion people. To address this problem, a genetic approach called biofortification which aims at enrichment of food grains with micronutrients has been evolved and used. Selection of genotypes for higher grain zinc content using marker assisted selection in rice is an effective strategy to address widespread dietary deficiency in human populations. In the present study fifty rice genotypes were subjected to molecular characterization using 7 SSR marker and 3 candadidate gene markers for grain zinc content. The clear and unambiguous bands obtained were scored and used for analysis. Polymorphism information content (PIC) ranged from 0.331 to 0.375. The genetic similarity matrix generated from the primer combinations was used to perform cluster analysis using the UPGMA clustering method where in the genotypes grouped under different clusters indicated the presence of diversity at molecular level as the genotypes showed similar banding pattern for various primers used. The single marker analysis was carried out to find the association between marker and the trait. Out of the seven polymorphic markers obtained, the markers viz., OsZIP3b, OsZIP4b and RM 23 exhibited P- value of 0.049, 0.046 and 0.038 respectively and corresponding R^2 (phenotypic variance) value of 6.2, 3.3 and 3.6 respectively indicating there is association between these markers and grain zinc content. Therefore, these markers can be used as a tool for identifying and mapping of new high zinc content genes in rice and also in biofortification program to improve grain zinc content.

Key words: *Zinc, SSR markers, Candidate gene markers, Biofortification, Single marker analysis*

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INTRODUCTION

Iron and Zinc are important elements for plant development, out of the 16 essential elements needed for plant growth, iron is used for the synthesis of chlorophyll and is essential for the function of chloroplasts. Zinc is involved in membrane integrity, enzyme activation and gene expression. In addition to being essential to plants, these are also an essential mineral nutrient for human beings. Micronutrient malnutrition is a serious health problem worldwide, affecting more than three billion people. There is evidence that the number of people and the proportion of global population suffering from micronutrient malnutrition have increased over the last four decades[3].

Rice is a staple food consumed in large quantities everyday by malnourished poor. Therefore, addition of even small quantities of micronutrients is beneficial. In the last two decades, new research findings generated by the nutritionists have brought to light the importance of micronutrients, vitamins and proteins in maintaining good health, adequate growth and even acceptable levels of cognitive ability apart from the problem of protein energy malnutrition. Once rice is biofortified with vital nutrients, the farmer can grow indefinitely without any additional input to produce nutrient packed rice grains in a sustainable way. This is the only feasible way of reaching the malnourished population. Furthermore, the use of Zn and Fe-dense grains results in greater seedling vigour and increased crop yields when the seeds are sown in micronutrient poor soils. To address this problem, a genetic approach called biofortification which aims at enrichment of food grains with micronutrients has been evolved and used[12].

To improve nutritive value of rice the preliminary step is to characterize genetic variability for grain Fe and Zn content in germplasm lines and then to use this variability for breeding nutrient rich rice[6]. Molecular marker assisted breeding is thus most suitable for intensive screening of large populations for identification of environment stable high grain iron and zinc rice genotypes. The genetic basis of accumulation of micronutrients in the grains will provide the basis for preparing the strategies and improving grain micronutrient content through marker assisted selection. DNA markers which are closely linked with desired traits allow the selection of plants possessing those traits prior to trait expression. Earlier reports of Chandel *et al*.[4], and Zhang *et al*.[16], have cited that grain zinc content in rice is governed by a number of QTL located on different regions of the chromosome with different phenotypic effects and can be assessed by using DNA markers that are linked to the trait. Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species. Information regarding genetic variability at molecular level could be used to help, identify and develop genetically unique germplasm that compliments existing cultivars.

Association or linkage disequilibrium (LD) mapping, which revolutionized genetic mapping in humans and is increasingly being applied to plants and is considered an efficient way of determining the genetic basis of complex traits. Comparing traditional linkage mapping which depends on restricted allelic variation with a small number of recombination events, using the association mapping there is no need to develop segregating populations such as F_2, double haploid or back-cross populations, instead a natural collection of inbred lines or varieties can be used. Association mapping involves searching for genotype to phenotype correlations among individual trait. It's high resolution is accounted for by the historical recombination accumulated in natural populations and collections of landraces, breeding materials and varieties. By exploiting broader genetic diversity, association mapping offers three main advantages over linkage mapping: mapping resolution, allele number and time saving by establishing a marker-trait association. Association mapping is therefore feasible, and potentially very useful in rice[7].

MATERIAL AND METHODS

The estimation of grain zinc content was carried out by XRF (X- Ray Fluorescence Spectrometry) at Indian Institute of Rice Research, Hyderabad. All the rice genotypes were estimated for both grain iron and zinc content while the molecular characterization was carried out only for grain zinc content using specific primers for zinc.

Fifty rice genotypes were subjected to molecular characterization using 7 SSR marker and 3 candadidate gene markers for grain zinc content. The information on markers used in the present investigation is provided in Table 1. The detailed procedures followed are elaborated as below.

DNA extraction

The DNA was extracted from the 50 genotypes by following CTAB extraction method[14], with required modifications.

- Two grams of fresh young leaves were harvested and the surface was cleaned with wet paper towel. The sample was ground to fine powder using liquid nitrogen with pre-chilled pestle and mortar.
- The powder was transferred into a sterile 2.0 ml eppendorf tube. Then 1 ml of pre-warmed (65 ^{0}C) CTAB extraction buffer was added. The solution was kept in water both at 65 ^{0}C for 10-15 min with intermittent mixing.
- The tubes were kept for 10 min to allow them to return to room temperature.
- Equal volume of chloroform : Isoamyl alcohol (CIA) (24:1) solution was added to the tubes and gently mixed by inverting.
- The solution was centrifuged at 15000 rpm for 10-15 min at room temperature.
- The aqueous supernatant solution was pipetted out and transferred to a fresh tube.
- DNA was precipitated by adding equal volume of ice cold isopropanol to the aqueous phase.
- The solution was left for overnight incubation at freezing temperature to enhance precipitation
- Solution was centrifuged at 15000 rpm for 5-10 minutes at 4°C.
- Supernatant was discarded (keeping pellet undisturbed).
- The DNA pellet was washed with 70 per cent alcohol (400 µl) for 30 min and the tubes were allowed to drain and dried at room temperature for 2-3 h.
- Then DNA was suspended in 50 µl TE (Tris EDTA) buffer (10 mMTrisHCl + 1mM EDTA + 2M NaCl, pH 7.5) in sterile eppendorf tube and kept for 45 min at room temperature.
- When DNA was fully dissolved, 1 µl of RNase (10 mg/ml) was added and incubated at 37 °C for one hour at room temperature.

Polymerase chain reaction

- Random primers: The decamers were used for the study
- Template DNA: crude genomic DNA extracts of different genotypes.
- dNTPs: The dNTP mix with concentration of 2.5mM each was obtained from Xcelris Genomics private ltd Ahmedabad, Gujarat, India.
- Taq DNA polymerase: Taq DNA polymerase (5U/µl) and 10 X Taq assay buffer were obtained from Xcelris Genomics private ltd Ahmedabad, Gujarat, India.
- Chemicals: Analytical grade chemicals were obtained locally
- Thermocycler - Eppendorf thermal cycler gradient was used for cyclic amplification of DNA.

The PCR amplification profile for SSR and Candidate gene markers

Sl. No.	Step	Temperature (^oC)	Duration	Number of cycles
1	Initial denaturation	94	5.00 min	1
2	Denaturation	94	1.00 min	
3	Annealing	55 (For SSR) 60 (Candidate gene marker)	0.45 sec	40
4	Extension	72	1.00 min	
5	Final extension	72	5.00 min	1
6	Dump	4	-	-

After the completion of the PCR, the products were stored at 4^0C until the gel electrophoresis was done.

Separation of amplified products by Agarose Gel

The amplified products from each tube along with 2µl of loading dye (bromophenol blue) were separated on 2.5 per cent agarose gel containing ethidium bromide at 70 volts (<5volts per cm of gel) using 1X TAE buffer of pH 8.0. Lambda DNA double digest was used as DNA molecular weight marker. The gel was photographed by using documentation system (Bio Rad).

Association analysis

The single marker analysis was done with paired 't' test and regression analysis using SPSS 16.0 to find the association between marker and the trait. The significant marker and grain Zinc content associations were indicated by a P-value (<0.05) with corresponding R^2 value. The R^2 value for each marker is the total phenotypic variation for a trait that is accounted by markers. It is calculated using following formula-

% R^2 = (Between group SS/ Total SS) × 100

The result is statistically significant if p-value is less than 0.05.Therefore the conclusion is that, there is an association between trait and marker.

RESULTS AND DISCUSSION

The phenotypic data of grain zinc content analyzed by XRF revealed that it ranged from 13 ppm to 23 ppm with a mean value of 16.77 ppm.

Out of 7 SSR primers used, four primers *viz*., RM 23, RM 35, RM 217 and RM 490 showed polymorphism while all the three candidate gene primers *viz*., OsZIP3b, OsZIP4b and OsZIP8a showed polymorphism. The clear and unambiguous bands obtained were scored and used for analysis. Polymorphism information content (PIC) value is reflection of allele diversity and frequency among the cultivars. PIC value of each marker can be evaluated on the basis of its alleles. The higher the PIC value of a locus, the higher the number of alleles detected as described by Lapitan *et al*.[11], In the present study, PIC value varied significantly for all the studied polymorphic markers, the level of polymorphism among the 50 genotypes was evaluated by calculating PIC values for each of the markers. The PIC values ranged from 0.331 (RM 23) to 0.375 (Os ZIP4b). This indicated that the genotypes used in the present study were more diverse due to differences in zinc content. Presence of polymorphism between genotypes revealed that the presence of genetic diversity at molecular level which implies that there is a ample scope to utilize the material in breeding programme. These results corroborate the earlier findings of Kumar *et al*.[9], and Kundur *et al*.[10]. The marker allele size range and PIC for different markers is shown in Table 2. Molecular profiles generated with the markers *viz*., OsZIP3b, OsZIP4b, OsZIP8a and RM23 are shown in Fig 1, Fig 2, Fig 3 and Fig 4 respectively.

Cluster Analysis

The genetic similarity matrix generated from the five SSR primer combinations was used to perform cluster analysis using the UPGMA clustering method following the Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis module of NTSYS[13]. Fifty rice genotypes were grouped into thirteen clusters at 0.68 Jaccard's similarity coefficient. Cluster 13 was the largest comprised of thirteen genotypes which was further sub divided into two clusters, 13(a) and 13(b), with seven and six genotypes each, respectively. Cluster 5 had eleven genotypes which was further sub divided into two clusters 5(a) and 5(b) with nine and two genotypes each, respectively. Cluster 10 had six genotypes, Cluster 3 had four while Cluster 2 and cluster 1 had three genotypes each. The Clusters 4, 7, 11 and 12 had two genotypes each whereas the clusters 6, 8 and 9 were solitary clusters. The clustering of genotypes for SSR profile is shown in Fig. 5. Cluster analysis by using candidate gene markers has revealed that eight clusters were formed at genetic similarity level of 0.61. Cluster 4 comprised with least genotypes of three, Cluster 1 had four genotypes while cluster 5 and Cluster 3 had five genotypes each. Cluster 2 had six genotypes while clusters 6, 7 and 8 had nine genotypes each. Clusters of candidate gene markers are shown in Fig.6. The genotypes grouped under different clusters indicate the presence of diversity at molecular level as the genotypes showed similar banding pattern for various primers used. The results were found similar to Fitzgerald *et al*.[5], who reported that the cluster analysis method was suitable for using the information derived from the markers to group the rice genotypes.

Marker and trait association

Out of the seven polymorphic markers obtained the markers OsZIP3b, OsZIP4b and RM 23 exhibited P- value of 0.049, 0.046 and 0.038 respectively and corresponding R^2 value of 6.2, 3.3 and 3.6 respectively (Table 3). Since the P- value of these three markers is less than 0.05 these are statistically significant and there is a significant association between the markers OsZIP3b, OsZIP4b and RM 23 with that of grain Zinc content.

Understanding the genetic basis of accumulation of micronutrients in the grains and mapping of the quantitative trait loci (QTL) will provide the basis for devising the plant breeding strategies and for improving grain micronutrient content through marker-assisted selection. Advances in the techniques of molecular biology have greatly improved the efficacy of breeding programs; currently molecular markers are used as key tools for genetic mapping of important traits and helps in breeding process by marker assisted selection. DNA markers that are closely linked with desired traits allow the selection of plants possessing those traits prior to trait expression. It is reported that grain Zn content in rice is governed by a number of QTLs located on different regions of the chromosome with different phenotypic effects[17]. Marker assisted selection for genotypes with traits of higher Zn content in rice grain is an effective strategy to address widespread dietary deficiency in human populations. Hence, QTLs responsible for this trait can be identified with closely linked molecular markers. Thus, to facilitate this approach preliminary steps are required to characterize molecular markers linked to QTLs for grain Zn content and study their phenotypic variation[15].

In single-marker analysis, the basic principle is that the genotype of a marker should be correlated with genotype at a linked QTL, i.e., each marker itself assumed as a potential QTL and the marker should also show a statistical influence on the trait. Out of the seven polymorphic markers obtained, the markers *viz*., OsZIP3b on chromosome 4, OsZIP4b on chromosome 8 and RM 23 on chromosome 1 exhibited P- value of 0.049, 0.046 and 0.038 respectively and corresponding R^2 value of 6.2, 3.3 and 3.6 respectively. Since the P- value of these three markers is less than 0.05 these are statistically significant and there is a significant association between the markers OsZIP3b, OsZIP4b and RM 23 with that of grain Zinc content. This study can be confirmed by

reports of Anuradha *et al*.[1], who reported 14 markers located on chromosomes 1, 2, 3, 4, 5, 6, 7 and 10 were linked with grain Zn concentration. Out of 14, 4 markers on chromosomes 3 (RM231, RM514), 6 (RM541) and 10 (RM484) explained high phenotypic variance significant at 1% (p=0.006 to 0.009). Similarly, Indurkar *et al*.[8], reported three markers RM12796 on chromosome 2, RM2489 on chromosome 3, RM287 on chromosome 11 significantly showed association with grain zinc content with a phenotypic variation of 15, 4, and 11%, respectively among the RIL population indicating many QTL's controlling the grain zinc content. The grain zinc content associated with SSR markers (RM152, RM263 and RM21) with 6.1 to 11.7% phenotypic variability were reported by Bekele *et al*.[2], This study shows that the marker and trait association analysis is an important strategy which helps in selection of primers for its utilization in marker assisted breeding programmes aimed at biofortification in rice. The association can be made more stringent by further analysis of more number of lines with the reported markers for both germplasm lines and segregating populations. The knowledge of QTL analysis and the information on DNA in identical genes on zinc accumulation is helpful for the identification of interesting alleles of relevant genes.

Table 1. List of DNA markers used for characterization of rice genotypes for grain zinc content

Marker	Sl. No.	Primers Name	Sequence (5'-3')	
			Forward	Reverse
SSR	1	RM490	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
	2	RM279	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG
	3	RM470	TCCTCATCGGCTTCTTCTTC	AGAACCCGTTCTACGTCACG
	4	RM23	CATTGGAGTGGAGGCTGG	GTCAGGCTTCTGCCATTCTC
	5	RM35	TGGTTAATCGATCGGTCGCC	CGACGGCAGATATACACGG
	6	RM21	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG
	7	RM217	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC
Candidate gene markers	1	OsZIP3b	CCTGCTGAGGCTGAGTTGAA	CGAGAACAAAGTAACAGGCTGC
	2	OsZIP4b	TGAGCTCATCATCACCAACCGTC	CACCTCCACCATCAAGGACG
	3	OsZIP8a	ATGAGGACGAACACCACCAC	CGGAGGGAGGGAGTAGTAATG

Table 2. Marker allele size range and PIC for different markers

Sl. No.	Primers	Molecular marker allele Size Range (bp)	Polymorphic Information Content value
1	OsZIP3b	370-390	0.354
2	OsZIP4b	660-680	0.375
3	OsZIP8a	880-910	0.371
4	RM 23	145-160	0.331
5	RM 35	200-240	0.372
6	RM 217	130-140	0.374
7	RM 490	100-120	0.372
	Mean	-	**0.364**

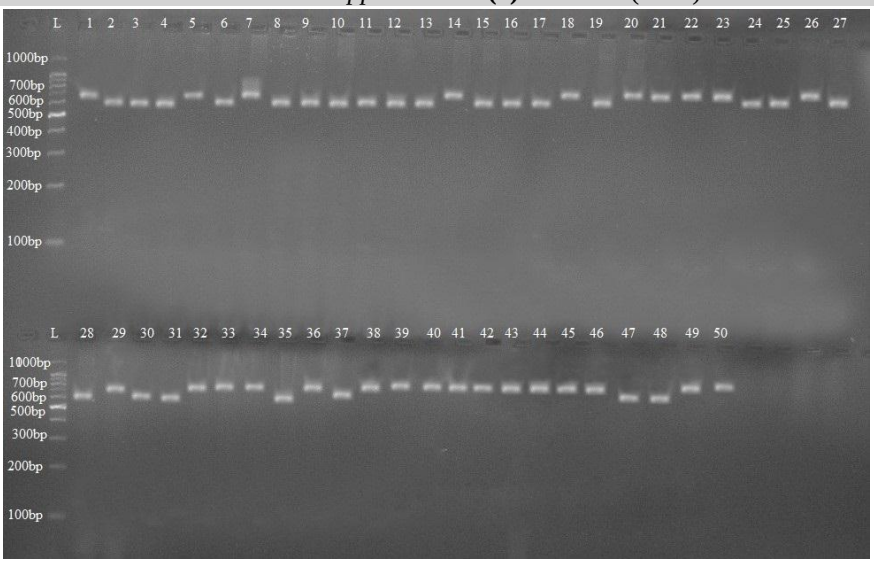

Fig. 1. Molecular profiling of OsZIP3b marker in 50 genotypes

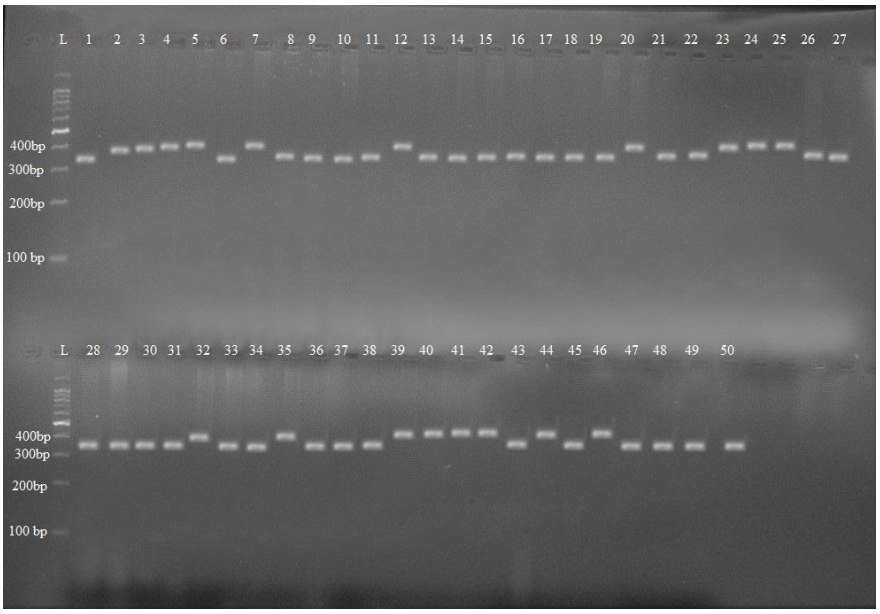

Fig. 2. Molecular profiling of OsZIP4b marker in 50 genotypes

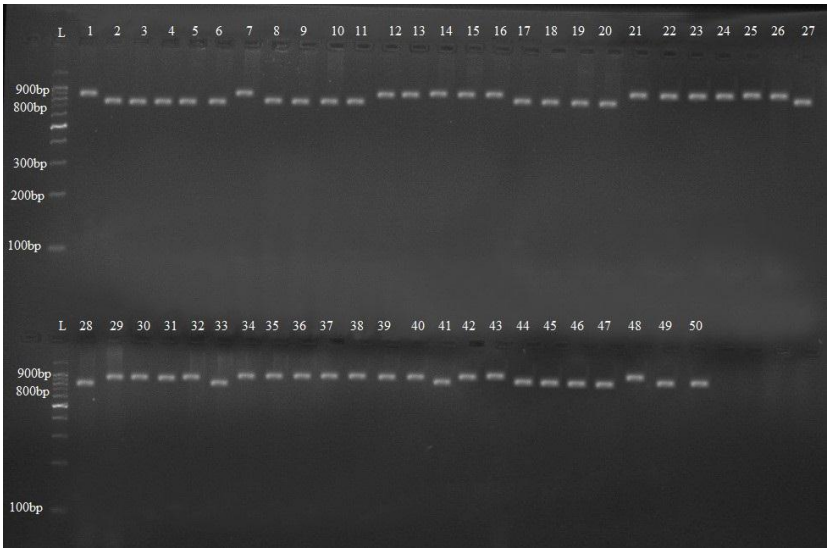

Fig. 3. Molecular profiling of OsZIP8a marker in 50 genotypes

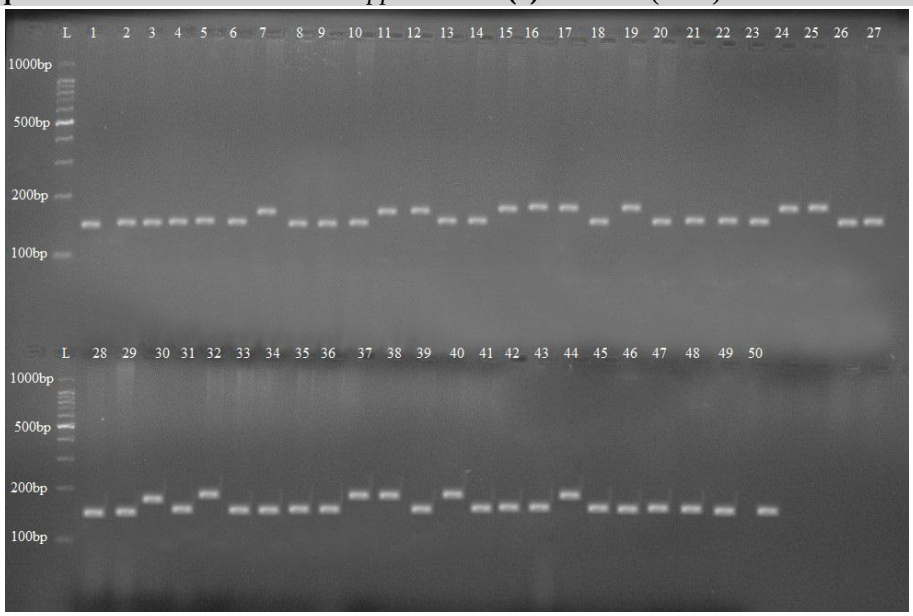

Fig. 4. Molecular profiling of RM23 marker in 50 genotypes

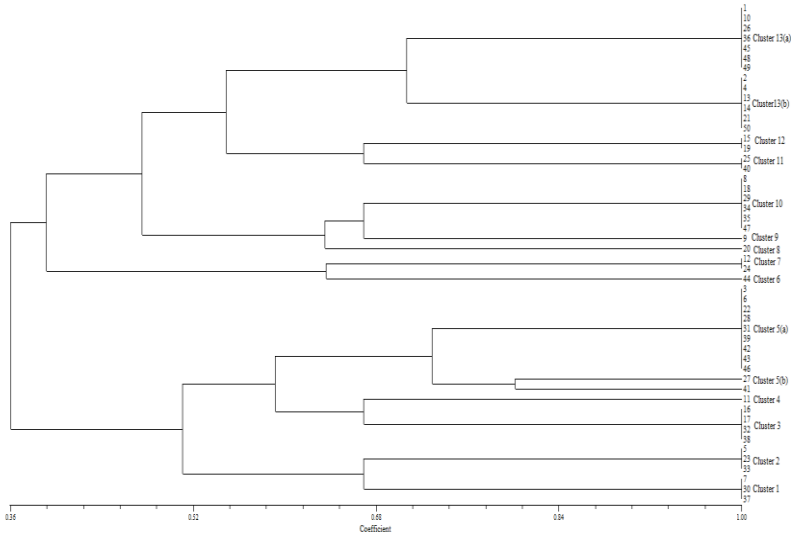

Fig. 5: Dendrogram showing genetic similarity among 50 rice genotypes using Jaccard Coefficient for SSR markers

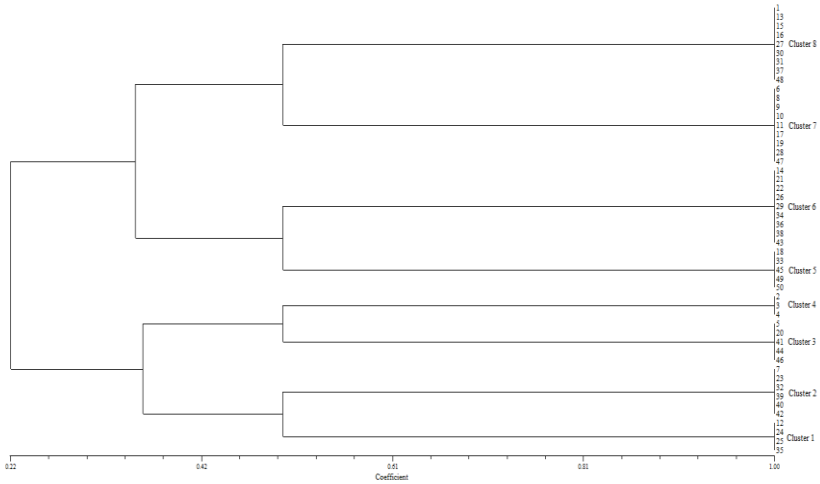

Fig. 6: Dendrogram showing genetic similarity among 50 rice genotypes using Jaccard Coefficient for candidate gene markers

Table 3. Association between markers with grain Zn content

MARKER	P- value	R^2 Value (%)	Marker and Trait Association
RM 490	0.308	-	No Association
RM 217	0.394	-	No Association
RM 23	0.038*	3.6	Associated
RM 35	0.092	-	No Association
OsZIP3b	0.049*	6.2	Associated
OsZIP4b	0.046*	3.3	Associated
OsZIP8a	0.238	-	No Association

CONCLUSION

The result of the present work represents the approach in understanding the biofortification of zinc in cereals like rice because trace minerals are important not only for human nutrition but also for plant nutrition. Plant-breeding with Marker Assisted Selection holds great promise for making a significant, low-cost and sustainable contribution to reducing deficiencies of micronutrients in humans. The single marker analysis carried out in the study successfully exhibited the association between marker and the trait. The molecular markers *viz*., OsZIP3b, OsZIP4b and RM 23 exhibited significant association with that of grain zinc content. Therefore, these markers can be used as a tool for identifying and mapping of new high zinc content genes in rice and also can be successfully utilized in breeding program for improvement of grain zinc content in rice.

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