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Article

Molecular characterization of rice genotypes for Zinc biosynthetic gene(s) using microsatellite simple sequence repeat (SSR) markers

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Abstract: Zinc deficiency is prevalent and affects nearly two billion people in the developing world, where mainly cereals are consumed as staple food by the people. It is essential to study the genetic characters of the rice genotypes containing genes responsible for zinc synthesis so that they can be used as breeding resource to develop nutrient rich rice varieties. In this study, 46 rice *(Oryza sativa* L., 2n=24) genotypes were studied for their Zinc biosynthesis ability using 3 Rice Microsatellite (RM) markers (RM23, RM217 and RM35) linked to Zn content at Biotechnology Laboratory of Bangladesh Institute of Nuclear Agriculture (BINA) and Genetics and Plant Breeding Laboratory of Bangladesh Agricultural University, Mymensingh during 5 August, 2013 to 30 April, 2014. The DNA samples were isolated by CTAB mini-prep method from 21 days older rice leaf samples followed by Polymerase chain reaction (PCR). Three markers (Rm23, RM35 and RM217) showed effective polymorphism in DNA band appearance for Zn content out of ten SSR markers. The alleles were separated on Polyacrylamide Gel Electrophoresis (PAGE) system. After molecular analyses of DNA bands using Power Marker software, variation was found in allele number, allele frequency, polymorphism information content (PIC) and gene diversity for each genotype. Number of alleles (23, 19 & 13), PIC-polymorphism information content (0.946, 0.911 & 0.829), gene diversity (0.948, 0.917 & 0.846), rare allele (16, 12 & 8) and null allele (0, 16 & 2) were observed for 3 rice microsatellite (RM) markers viz. RM35, RM217 and RM23, respectively for the 46 rice genotypes. A total of 55 alleles were found by using 3 SSR primers for all genotypes. The number of alleles per locus ranged from 13 to 23 with an average of 18.33 out of total 55 alleles. The UPGMA Dendrogram categorized all the varieties into 7 distinct clusters based on 50% of average genetic distance among the 46 genotypes (Figure 2 and Table 7). Since the primers showed almost similar and highest PIC values, based on this study, the larger range of similarity values using SSR markers will provide greater confidence for the assessment of genetic relationships among the varieties. The information obtained from SSR profiling helped in identifying the varieties containing genes for Zn synthesis among the selected rice genotypes. Among all the rice genotypes, Kumra Ghor, Ghigoj, Tilek Kuchi contained high Zn content found in another chemical analysis. Most of the genotypes contained candidate gene for Zn synthesis and can be used as breeding materials to develop nutrient (Zn) rich rice varieties through different selective breeding methods in future.

Keywords: bio-fortification; gene bank; gene diversity; genotype; polymorphism

1. Introduction

Rice is a staple food crops cultivated worldwide and feeds more than one half of the world's population. Humans require at least 49 nutrients for their normal growth and development, and the demand for most nutrients is supplied by cereals, particularly rice due to its staple role (Welch and Graham 2004). The lack of

micronutrients such as Zn in staple food crops is a widespread nutrition and health problem in developing countries. It is an essential micronutrient for human diet and their deficiency causes various problems in human body like retardation of growth in children, male reproduction, low blood sugar, poor bone growth, brain disorders, high blood cholesterol, poor circulation, eating disorders, problems with female reproduction, poor skin, nails and hair deformations and poor sense of taste and smell (Stewart 2010). Identification as well as development of rice varieties with higher amount of micronutrient (Zn) is an important way to overcome this malnutrition. Around two billion people suffer from nutrient deficiency, while prevalence of Zn deficiency is much harder to quantify due to the lack of a reliable and easy clinical assay (FAO 2004). Many researchers have already studied genetic variation for mineral elements in cereal grains such as rice (Gregorio *et al.* 2000; Zhang *et al.* 2004), wheat (Cakmak *et al.* 2000). The rapid development of molecular technology provides great opportunities to enhance nutritive values of traditionally cultivated crops. One of the strategies is "Biofortification" which is defined as the enhancement of micronutrient levels of staple food crops through biological processes, such as plant breeding and genetic engineering (Bouis 2002). It is the best option available to alleviate nutrient disorders among the population of Bangladesh. In addition to agronomical management, selection of genotypes with high efficiency of Zn accumulation in the endosperm, aleurone layer and their bioavailability from existing germplasm may be an efficient and reliable way to deliver Zn nutrition benefits to farmers and local population. Some wild relatives of rice were found to have higher grain Zn concentrations compared with the cultivated rice germplasm. These can be used as breeding materials for biofortification purpose. DNA fingerprinting/profiling is being used as versatile tools for investigating various aspects of plant genomes including characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding (Cheng *et al.* 2009). Plant breeders may be involved in breeding staple food crops with more Zn content need to identify donor parents carrying the target traits. So, identification of rice genotypes containing gene for Zinc synthesis and their characterization is essential to develop Zinc rich rice variety in order to supply Zinc nutrient to the people through their staple food like rice.

2. Materials and methods

2.1. Experimental site, duration and selected genotypes

Seeds of all the selected 46 local rice varieties (Table 1) with diverse genetic background were collected from the Gene Bank of Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. All accessions were grown in the experimental field of Biotechnology and Plant Breeding division of BINA and leaf samples collected from these varieties were used for molecular analysis. The molecular characterizations were done using rice leaf sample in the Genetic Fingerprinting Laboratory of BINA and Bangladesh Agricultural University (BAU), Mymensingh.

2.2. Raising of seedling, collection of leaf sample, seedling transplanting and seed harvesting

Ten to fifteen seeds of each variety were selected randomly and dried in oven at 54°C for 48 hrs for better germination. After germination in the wet blotting paper in petridishes, the seedlings were grown in small plastic tray. After 21 days, 4-5 leaf samples were collected from the young plants of all the genotypes followed by washing with 70% alcohol and ddH_2O to avoid any contamination or infection by the germs and subsequently stored at –20°C after packing into sterile polythene bag with proper labeling separately for further isolation of genomic DNA.

2.3. Isolation of rice genomic DNA

The genomic DNA was extracted from 21 day old/young leaves using N-Cetyl-N, N, N-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990; IRRI, 1997). Leaf piece was cut into 3-4 small pieces in the well of a Mortar (Thomas Scientific, USA) and 270µL of extraction buffer (Solution: Tris-50 mM pH=8.0, EDTA-25 mM, NaCI-300 mM, 20% SDS and deionized water) was added. The leaf tissue was crushed using an alcohol sterilized blunt ended pestle. It was ensured that the leaf pieces were completely ground and 400µL more of extraction buffer was added to the well containing the homogenized leaf sample. The entire content was transferred from the well of Mortar into 1.5 mL capacity eppendorf tube. Then the mixture was vortexed for 20 seconds and incubated for 10 minutes at 65^0C in the hot water bath. Then 100 µL 5M NaCl and 100 µL CTAB solution were added and inverted gently to suspend the samples evenly. Again, the mixture was vortexed for 20 seconds and incubated for 10 minutes at 65^0C in the hot water bath. About half volume (400-900µL) of PCI (Phenol: Chloroform: Isoamyl alcohol= 25:24:1, pH=8.0) was added. The samples were spinned down at

12000 rpm for 15 minutes into a centrifuge machine. Then the supernatant was transferred into a new eppendorf tube and 600 µL ice-cold isopropanol was added to the supernatant and shakened well. The mixture was again spinned down at 12000 rpm for 15 minutes by centrifuge machine. The supernatant was discarded and the pellet was washed with 200 µL 70% ethanol. At last the sample was spinned down again at 12000 rpm for 5 minutes, then ethanol was removed carefully, the pellet was left for air-drying over night at room temperature with the tube cap open. After complete drying of pellet, depending on the size of the pellet, about 50-100 µL of sterile T.E. buffer (10 mM Tris-HCl, pH 8.0 and 1mM EDTA) or sterile distilled water was added to the tube for dissolving the pellet. Finally, the DNA samples were stored at - 20°C.

2.4. Confirmation, quantification and optimization of DNA concentration

The amount of genomic DNA was quantified at 260nm spectrophotometrically at Professor Muhammad Hossain Central Laboratory of BAU, Mymensingh, Bangladesh. Presence of genomic DNA was confirmed on 2% agarose gel qualitatively. Using the absorbance reading obtained for DNA sample of each rice variety, the original DNA concentrations were determined and adjusted to 25 ng/µL for further PCR analysis.

$$\text{DNA conc. (µg/µl)} = \left[\text{Absorbance} \times \frac{\text{Vol. of deionized water (µl)}}{\text{Amount of DNA (µl)}} \times \text{conversion factor } (0.05) \times 1000 \right]$$

2.5. Identification and selection of SSR markers

A set of ten rice microsatellite markers (RM9, RM21, RM23, RM35, RM167, RM180, RM217, RM296, RM400 and RM520) distributed in the rice genome for Fe and Zn content were identified from the available data-based search (http://www.gramene.org/) for rice SSR markers as described by Panaud *et al.* (2003), Akagi *et al.* (2005), Temnykh *et al.* (2000), McCouch *et al.* (2002) and Shankar Ilango and N Sarla (2010).

2.6. PCR amplification and electrophoresis of sample DNA

These microsatellite primers were purchased from commercially available microsatellite primer kits (Merck Bioscience, USA). Individual PCR amplifications for each microsatellite were performed in a oil free and programmable thermal controller (BioRad, California, USA). The PCR protocol involved a total volume of 10 µL reaction mixture (Sambrook *et al.*, 1989) containing 2 µL of genomic DNA and 8 µL of PCR cocktail (1.0 µL dNTP mix, 0.6 µL of $MgCl_2$, 1.0 µL Taq buffer (B), 3.20 µL ddH_2O, 1.0 µL forward and 1.0 µL reverse primer and 0.2 µL of *Taq (Thermophilus aquaticus)* DNA polymerase). The basic PCR program to amplify DNA was as follows: an initial hot start and denaturing step at 94°C for 5 min followed by 34 cycles of a 30 second denaturation at 94°C, a 30 second annealing at appropriate temperature (52 or 67°C) depending on the primer, and a 1 min primer elongation at 72°C. A final extension step at 72°C for 5 min was performed. PCR was confirmed by electrophoresis on 2% agarose gel.

2.7. Polymorphism survey

The selected 3 primers viz. RM23, RM35 and RM217 positioning in chromosome numbers 1, 1 and 6 of rice genome (Temnykh *et al.* 2001) with clear and expected amplified product sizes were selected and used for microsatellite analysis for Zn content among 46 rice varieties in the present study. The details of the primers are given in Table 2.

2.8. Electrophoretic separation and visualization of PCR products

Prior to electrophoresis, each PCR-product was prepared with 2µL loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA). Loading dye was used for monitoring the loading sample stayed in the gel well. The gel was carefully placed in the electrophoresis gel chamber keeping the gel horizontal and submerged in 1× TBE buffer. After loading of DNA sample into gel, a molecular weight marker DNA or ladder (25 bp) was loaded in one side of the gel. Electrophoresis was performed at 80 V, 500 mA for 2 hour and 30 minutes according to PCR product size of the selected marker. The DNA migrates from negative to positive electrode (black to red). The electrophoresis was stopped after the bromophenol blue dye had reached three-fourths of the gel length. PCR-products were electrophoresed on 8% polyacrylamide gel. After completing the electrophoresis, the gel with DNA samples was carefully taken out from the gel tank and stained with ethidium bromide solution (10mg/mL) for 12-14 minutes. For documentation, the gel was carefully taken out from ethidium bromide solution, washed and placed on the high performance ultraviolet transilluminator

(UVP, BioDoc-It™ System) at the wave-length of 302 nm to observe the quality of the genomic DNA. Then the gel was examined and photographed by using a Gel Doc camera and finally used in molecular analysis.

2.9. Scoring and analysis of microsatellite data

Microsatellite DNA profiles of all rice varieties against three primers are shown in Figure 2. Molecular weights for microsatellite products were also estimated with Alpha Ease FC 4 software from these DNA band images (Yeh *et al.,* 1999). A single genotypic data matrix was constructed for all loci. The summary statistics including the number of alleles per locus, major allele frequency, genetic diversity and polymorphism information content (PIC) values were determined by using Power Marker (version 3.23) (Yeh *et al.,* 1999). PIC provides an estimate of the discriminatory power of a marker by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles were observed. Estimation of Nei's genetic distance values (D) and construction of UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram was constructed using the software.

3. Results and Discussion

3.1. PCR amplification of all rice genotypes

PCR was done to check each sample for parental polymorphism using specific Rice Microsatellite (RM) primers and thermal profile (Table 3). Out of 10 primers, 3 RM primers showed polymorphism. Thirty percent polymorphism was found among the markers calculated using the following formula:

$$\text{Polymorphism}\,(\%) = \frac{\text{RM primers showing polymorphism}}{\text{The total number of RM primers used}} \times 100$$

3.2. Genotyping through molecular marker

Unlike morphological and biochemical markers, DNA markers are unlimited in numbers and are not affected by environmental factors and /or the developmental stages of the plant (Ovesna *et al.* 2002, Saker *et al.* 2005). The genetic markers arise from different classes of DNA mutations such as substitution mutation, rearrangements or errors in replication of tandemly repeated DNA. All the microsatellite loci (RM23, RM35 and RM217) amplified were found to be polymorphic. Molecular analyses were done using Power Marker (Version 3.25, Liu & Muse 2005) software where 3 primers identified 55 alleles across 46 varieties. The number of alleles ranged from 13 to 23 with average 18.33 per locus. The locus RM35 had the highest number of alleles (23) with maximum allele size difference (55) and the locus RM23 had the lowest number of alleles (13) with minimum allele size difference (25) (Table 4 & 5). Yang *et al.* (1994) found up to 25 alleles for 10 microsatellite markers among 238 accessions of *Indica* and *Japonica* cultivars and landraces.

3.3. Gene diversity, heterozygosity and availability

According to Nei's (1972), the highest level of gene diversity value (0.948) was observed in loci RM35 and the lowest level of gene diversity value (0.8y46) was observed in loci RM23 with a mean diversity of 0.904 (Table 5). It was observed that marker detecting the fewer alleles showed lower gene diversity than those detected higher number of alleles which revealed higher gene diversity. The maximum number of repeats within the SSRs was also positively correlated with the genetic diversity. Herrera *et al.* (2008) also observed that the gene diversity at each SSR locus was significantly correlated with the number of alleles detected number of repeat motif and with the allele size range. Among all the genotypes, 0.00 heterozygosity were observed for each of the marker and single gene was found for the responsive genotype against the selective SSR marker (Table 4).

3.4. Major allele

The allele with maximum frequency is known as major allele. RM23 showed major allele with highest frequency (0.261) and size 143bp while RM35 showed major allele with minimum frequency 0.087 and size 197bp. RM217 showed major allele with medium frequency 0.174 and size 0.00bp (Table 5).

3.5. Rare allele

The allele with frequency of less than 0.05 is known as rare allele. Maximum rare alleles (16) were found for RM35 followed by RM217 (12) and RM23 (8) with average 12 rare alleles across 46 rice genotypes for three markers (Table 5).

Table 1. List of experimental rice genotypes with their source of collection.

SL. No.	Genotypes	Source of collection	SL. No.	Genotypes	Source of collection
G1	Rupessor	BINA Gene Bank	G24	Chini Shail	BINA Gene Bank
G2	Karengal	BINA Gene Bank	G25	Pengek	BINA Gene Bank
G3	Kalomota	BINA Gene Bank	G26	Gota	BINA Gene Bank
G4	Nona Kochi	BINA Gene Bank	G27	Kherail	BINA Gene Bank
G5	Vashiara	BINA Gene Bank	G28	Lal40	BINA Gene Bank
G6	Patnai Balam	BINA Gene Bank	G29	Sali	BINA Gene Bank
G7	Tilek Kuchi	BINA Gene Bank	G30	Gotamala	BINA Gene Bank
G8	Bashful Balam	BINA Gene Bank	G31	Rani Shalot	BINA Gene Bank
G9	Chap Shail	BINA Gene Bank	G32	Dhar Shail	BINA Gene Bank
G10	Mohime	BINA Gene Bank	G33	Malagoti	BINA Gene Bank
G11	Mowbinni	BINA Gene Bank	G34	Volanath	BINA Gene Bank
G12	Lalanamia	BINA Gene Bank	G35	Kathi Goccha	BINA Gene Bank
G13	Gengeng Binni	BINA Gene Bank	G36	Khejur Chori	BINA Gene Bank
G14	Kumra Ghor	BINA Gene Bank	G37	Ghocca	BINA Gene Bank
G15	Kalmilata	BINA Gene Bank	G38	Asam Binni	BINA Gene Bank
G16	Hati Bajor	BINA Gene Bank	G39	Marish Shail	BINA Gene Bank
G17	Ponkhiraj	BINA Gene Bank	G40	Raja Shail	BINA Gene Bank
G18	Ghigoj	BINA Gene Bank	G41	Nunnia	BINA Gene Bank
G19	Hogla	BINA Gene Bank	G42	Bogi	BINA Gene Bank
G20	Bhute Shalot	BINA Gene Bank	G43	Khuchra	BINA Gene Bank
G21	Bajra Muri	BINA Gene Bank	G44	Shaheb Kachi	BINA Gene Bank
G22	Mondessor	BINA Gene Bank	G45	Golapi	BINA Gene Bank
G23	Jolkumri	BINA Gene Bank	G46	Sadagotal	BINA Gene Bank

Table 2. Details of the selected 3 SSR primers used for molecular analysis of rice crop for Zn content.

Primers	Sequence (5'-3')	PCR Product Size (bp)	Ta (^{0}C)	Chrom. position	Reference
RM23*	F=CATTGGAGTGGAGGCTGG R=GTCAGGCTTCTGCCATTCTC	145	55 55	1	Shankar Ilango and N. Sarla (2010)
RM217*	F=ATCGCAGCAATGCCTCGT R=GGGTGTGAACAAAGACAC	133	55 55	6	
RM35*	F=TGGTTAATCGATCGGTCGCC R=CGACGGCAGATATACACGG	207	55 55	1	Tenmykh *et al.* (2000)

Here, Ta-Annealing temperature, F-Forward sequence and R-Reverse sequence of the primer

Table 3. Thermal profile for polymerase chain reaction (PCR).

Step no.	Steps	Temperature (^{0}C)	Time	No. of cycle
1	Initial denaturation	94	5 min.,	1
2	Denaturation	94	30 sec.,	
3	Annealing	55	30 sec.,	34
4	Extension	72	1 min.,	
5	Final Extension	72	7 min.,	1
6	Cooling	4	α	

Table 4. Molecular analyses results found in all varieties for three markers.

Marker	Repeat motif	Sample size	No. of observation	Allele size ranges(bp)	Diff. (bp)	Hetero zygosity	Availability	f
RM23	(GA)15	46.0	46.0	122-147	25	0.00	1.00	1.00
RM35	(GA)19	46.0	46.0	152-207	55	0.00	1.00	1.00
RM217	(CT)20	46.0	46.0	90-136	46	0.00	1.00	1.00
Mean	------	46.0	46.0	---------	42	0.00	1.00	1.00

Table 5. Size and frequency of alleles and diversity index at 3 SSR loci across 46 rice varieties.

Locus	Major allele		Genotype No.	No. of alleles	Rare alleles	Null allele	PIC	Gene Diversity	SMM Index
	Size (bp)	Freq. (%)							
RM23	143	0.261	13.00	13.00	8.0	2.00	0.829	0.846	0.00
RM35	197	0.087	23.00	23.00	16.0	0.00	0.946	0.948	0.00
RM217	0.00	0.174	19.00	19.00	12.0	16.00	0.911	0.917	0.00
Total	340	0.522	55.00	55.00	36.0	18.00	0	2.711	0.00
Mean		0.174	18.33	18.33	12.0	6.00	.895	0.904	0.00

Table 6. Number of allele of different sizes with frequency, variance, standard deviation, 2.5%l.b and 97.5%u.b found at 3 SSR loci across 46 rice varieties.

RM23							RM35						
Allele size (bp)	Count	Freq.	Variance	SD	2.5% l.b.	97.5% u.b.	Allele size (bp)	Count	Freq.	Variance	SD	2.5% l.b.	97.5% u.b.
0	2	0.022	0.000	0.022	0.000	0.043	152	2	0.022	0.000	0.022	0.000	0.065
122	2	0.022	0.000	0.022	0.000	0.065	156	4	0.043	0.001	0.030	0.000	0.109
123	8	0.087	0.002	0.042	0.022	0.174	162	2	0.022	0.000	0.022	0.000	0.065
135	2	0.022	0.000	0.022	0.000	0.065	164	4	0.043	0.001	0.030	0.000	0.130
139	2	0.022	0.000	0.022	0.000	0.043	167	2	0.022	0.000	0.022	0.000	0.065
140	4	0.043	0.001	0.030	0.000	0.109	170	6	0.065	0.001	0.036	0.000	0.130
141	2	0.022	0.000	0.022	0.000	0.065	172	2	0.022	0.000	0.022	0.000	0.065
142	14	0.152	0.003	0.053	0.065	0.283	173	2	0.022	0.000	0.022	0.000	0.087
143	24	0.261	0.004	0.065	0.152	0.391	174	2	0.022	0.000	0.022	0.000	0.043
144	10	0.109	0.002	0.046	0.022	0.196	181	4	0.043	0.001	0.030	0.000	0.087
145	18	0.196	0.003	0.058	0.087	0.326	182	6	0.065	0.001	0.036	0.000	0.152
146	2	0.022	0.000	0.022	0.000	0.065	184	6	0.065	0.001	0.036	0.022	0.130
147	2	0.022	0.000	0.022	0.000	0.065	185	4	0.043	0.001	0.030	0.000	0.109
RM217							186	4	0.043	0.001	0.030	0.000	0.109
0	16	0.174	0.003	0.056	0.087	0.261	187	6	0.065	0.001	0.036	0.000	0.130
90	2	0.022	0.000	0.022	0.000	0.065	188	6	0.065	0.001	0.036	0.000	0.152
91	8	0.087	0.002	0.042	0.022	0.152	190	4	0.043	0.001	0.030	0.000	0.109
92	12	0.130	0.002	0.050	0.043	0.239	193	4	0.043	0.001	0.030	0.000	0.109
94	6	0.065	0.001	0.036	0.000	0.130	197	8	0.087	0.002	0.042	0.022	0.152
95	2	0.022	0.000	0.022	0.000	0.065	200	6	0.065	0.001	0.036	0.022	0.130
96	4	0.043	0.001	0.030	0.000	0.109	201	4	0.043	0.001	0.030	0.000	0.109
99	2	0.022	0.000	0.022	0.000	0.065	204	2	0.022	0.000	0.022	0.000	0.065
110	4	0.043	0.001	0.030	0.000	0.130	207	2	0.022	0.000	0.022	0.000	0.065
112	2	0.022	0.000	0.022	0.000	0.065	------	------	------	------	------	------	------
115	2	0.022	0.000	0.022	0.000	0.065	------	------	------	------	------	------	------
116	2	0.022	0.000	0.022	0.000	0.065	------	------	------	------	------	------	------
117	6	0.065	0.001	0.036	0.000	0.130	------	------	------	------	------	------	------
124	2	0.022	0.000	0.022	0.000	0.043	------	------	------	------	------	------	------
126	2	0.022	0.000	0.022	0.000	0.065	------	------	------	------	------	------	------
129	4	0.043	0.001	0.030	0.000	0.109	------	------	------	------	------	------	------
133	4	0.043	0.001	0.030	0.000	0.109	------	------	------	------	------	------	------
135	6	0.065	0.001	0.036	0.022	0.152	------	------	------	------	------	------	------
136	6	0.065	0.001	0.036	0.000	0.130	------	------	------	------	------	------	------

Table 7. List of rice genotypes into seven clusters.

Clusters	Genotypes found	Percent (%)	Genotypes
I	1	2.174	Nunnia
II	10	21.739	Asambinni, Chini Shail, Marish Shail, Ponkhiraj, Lalanamia, Pengek, Ghigoj, Hogla, Hati Bajor, Kalomota,
III	9	19.562	Kalmilata, Patnai Balam, Jolkumri, Nona Kochi, Rupessor, Tilek Kuchi, Vashiara, Karengal, Malagoti
IV	3	6.522	Kherail, Golapi, Lal40
V	4	8.696	Ghocca, Gota, Rani Shalot, Sadagotal
VI	2	4.348	Genegeng Binni, Mowbinni
VII	17	36.957	Gotamala, Kathi Goccha, Sali, BajraMuri, Bhute Shalot, Kumra Ghor, Mohime, Mondessor, Shaheb Kachi, Chap Shail, Khuchra, Dhar Shail, Bogi, Volanath, Raja Shail, Bashful Balam, Khejur Chori
Total	46	100.0	46 Rice genotypes

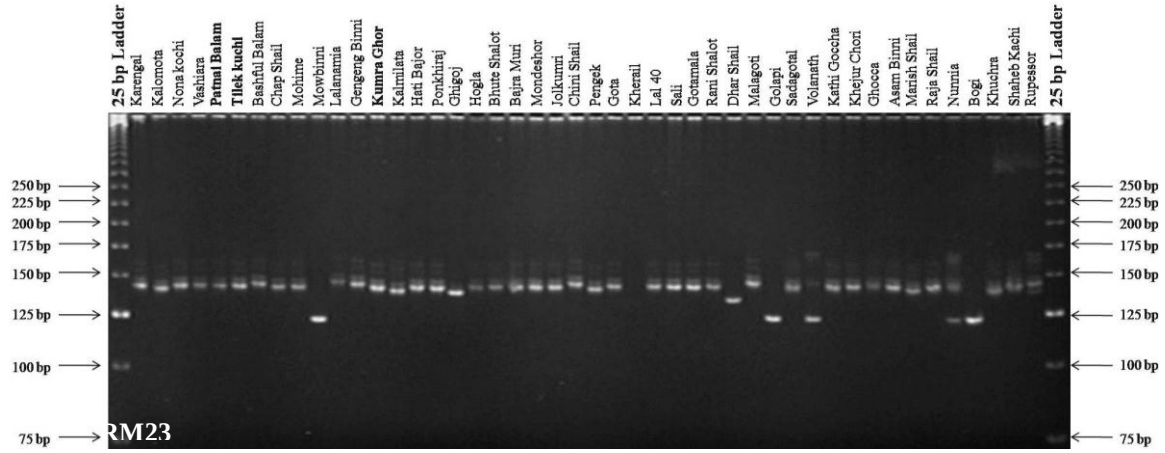

Figure 1a. DNA banding profiles of 46 rice varieties for RM23 where each consecutive lane represents a single variety.

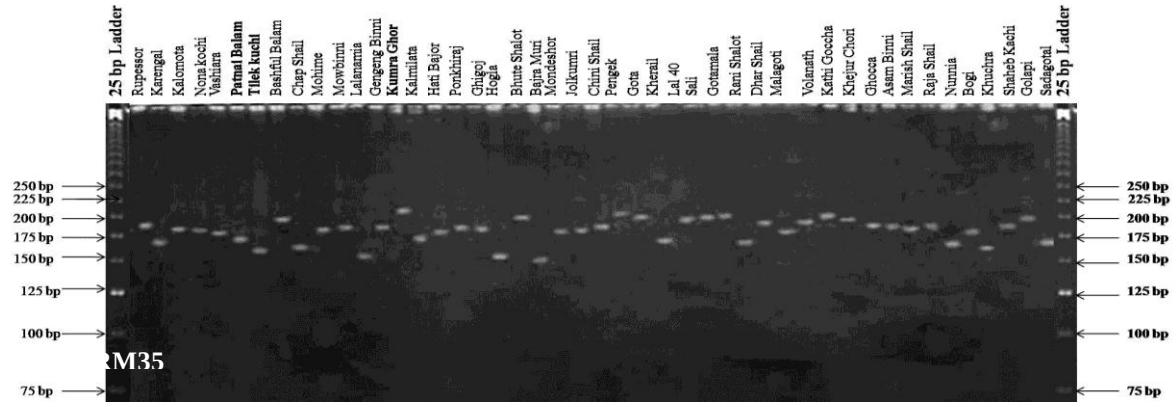

Figure 1b. DNA banding profiles of 46 rice varieties for RM35 where each consecutive lane represents a single variety.

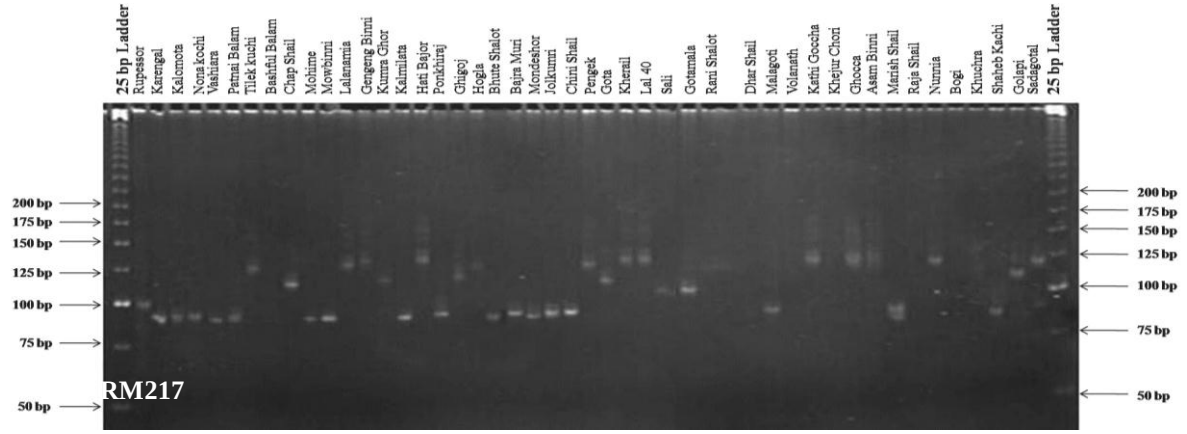

Figure 1c. DNA banding profiles of 46 rice varieties for RM217 where each consecutive lane represents a single variety.

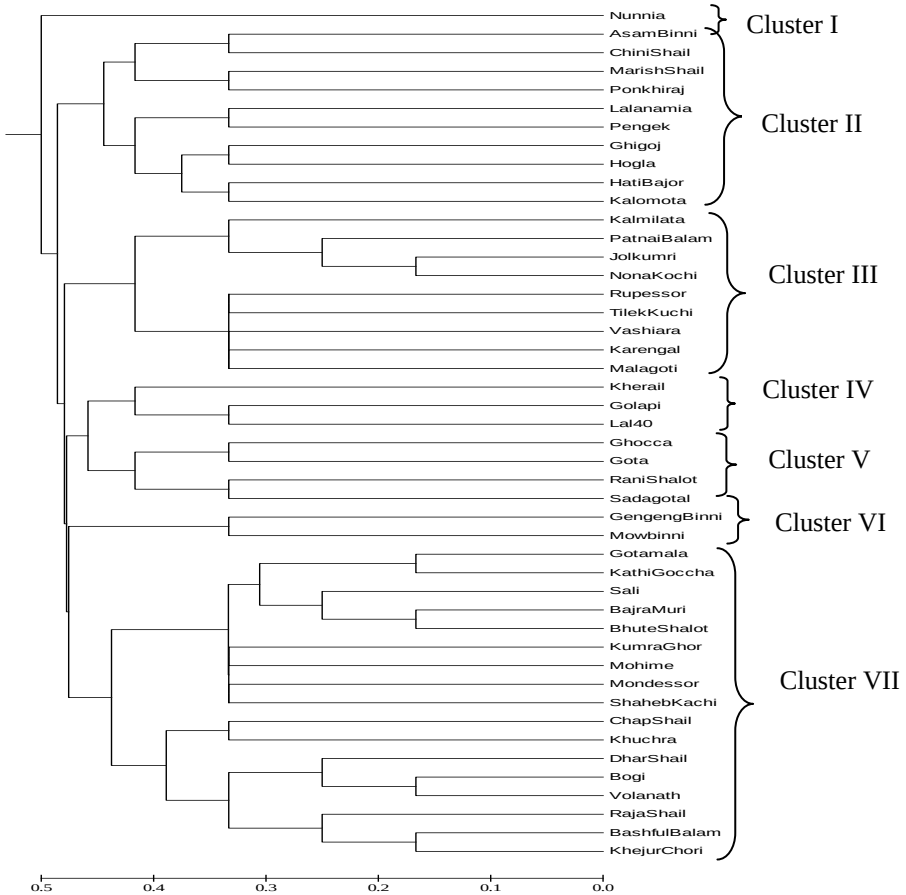

Figure 2. UPGMA Dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation among 46 rice genotypes according to SSR analyses (Sub cluster was cut at 50% of average Nei's genetic distance 0.45)

3.6. Null allele

Null allele means no appearance of allelic band in the genotype for the selected marker. Maximum null alleles (16) were found for RM217, no null alleles were observed for RM35 and RM showed 2 null alleles among 46 rice genotypes (Table 5).

3.7. Allelic diversity

Using 3 primers across 46 rice varieties, a total of 55 alleles were found in the present study of which RM35 showed highest number of alleles (23) followed by RM217 (19) and RM23 (13) in descending order (Table 5).

Rahman *et al.* (2006, 2008) reported 18 and 78 alleles respectively while analyzing with 3 primers (RM11; RM151 and RM153) & 5 primers (RM1; RM151; RM153; RM334 and RM335) in which PIC values were 0.670; 0.707; 0.698 & 0.862; 0.923; 0.831; 0.865 and 0.910 respectively. Yang *et al.,* (1994) found up to 25 alleles for 10 microsatellite markers among 238 accessions of *Indica* and *Japonic*a cultivars and landraces. The size variation between the smallest and the largest allele at a given SSR locus was correlated with the number of alleles per locus. Thus, RM23 presented the smallest allele size range (25bp), while RM35 presented the largest allele size range (55bp) and RM217 showed 46bp allele size range (Table 4).

3.8. PIC values

The PIC values are dependent on the genetic diversity of the cultivars chosen and this investigation had a high proportion of traditional varieties which would have the effect of increasing the PIC values. It is important to indicate that the selection by breeders have increased the frequency of the alleles or allelic combination with favorable effects at the expense of the others, eventually eliminating many of them. The markers in the specific chromosome and the allele size along with their frequencies and PIC values have been shown in the Table 5. The PIC values for 3 primers obtained in the present study varied from 0.829 for RM23 to 0.946 for RM35, with an average PIC value of 0.895 (Table 5). Among the markers used in this study RM35 showed higher PIC values and RM23 showed lower than the others. Lower PIC value may be the result of closely related genotypes and higher PIC values might be the result of diverse genotypes. The number of alleles amplified by a primer and its PIC values also depends upon the repeat number and the repeat sequence of the microsatellite sequences (Temnykh *et al.* 2000, 2001; Yu *et al.* 2003) showed that larger repeats and GA- repeats yield higher number of alleles and higher PIC values. Contrary to this, Temnykh *et al.* (2000) showed that (CTT), and AT-rich tri-nucleotide repeats amplified with higher efficiency and revealed greater polymorphism overall. RM35 having (GA)19 repeat motiff was a most informative microsatellite marker for this set of germplasm, as it yielded 23 alleles with PIC value 0.946 and gene diversity 0.948.

3.9. Banding pattern of 46 rice genotypes for 3 SSR markers

All the figures of banding patterns of 46 rice genotypes for molecular analysis of zinc content using three polymorphic SSR primers are presented in figure 1a, 1b and 1c. In this experiment, three polymorphic SSR markers *viz.*, RM23, RM35 and RM217 showed highly polymorphism among the 10 SSR primers. The RM35 showed good polymorphism than the other SSR markers used for screening of rice genotypes for zinc content.

3.10. Genetic distance between the genotypes

The values of pair-wise comparisons of Nei's (1972) genetic distance (D) between genotypes were computed from combined data for the 3 primers, ranged from 0.00 to 1.00. Higher genetic distance was observed between some lines pair than the other lines combination. The means of genetic distances between lines were used to evaluate the genetic diversity of different lines. Highly diversified lines (Kumra Ghor, Ghigoj, Tilek Kuchi etc.) carrying gene for Zn content could be useful in breeding program to have potential genetic gains.

3.11. UPGMA Dendrogram

Forty six rice lines used in the experiment were categorized by Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) (Figure 2). These rice lines have been differentiated into 7 main clusters based on the 50% of average genetic distance among 46 varieties. The clusters were separated into several sub-clusters. The dendrogram showed that the lines (Bashful Balam, Khejur Chori; Gotamala, Kaythi Goccha; Jolkumri, Nona Kochi etc.) were closely related belonging to the same cluster while the lines (Nunnia, Bashful Balam, Gengeng Binni, Rani Shalot, Golapi, Jolkumri) belonging to different cluster suggesting that these varieties were genetically diverse in origin. The dendrogram revealed that the lines that are derivatives of genetically similar placed in the same cluster.

3.12. Clustering of rice genotypes

Cluster analysis was performed using the UPGMA method to group the studied genotypes based on similarity coefficient. Mainly 7 clusters were formed at genetic similarity level of 0.0-1.0 (Figure 2). Cluster I, II, III, IV, V, VI and VII contained 1, 10, 9, 3, 4, 2 and 17 genotypes respectively (Table 7). Among the total genotypes those which showed comparatively same banding patterns were grouped into same cluster.

4. Conclusions

A large number of people have been suffering from Zinc deficient problems in Bangladesh. So, Bio-fortification of rice with highest Zn element is a vital work to supply Zn nutrient to the poorest people of Bangladesh. Plant-breeding with Marker Assisted Selection (MAS) holds great promise for making a significant, low-cost and sustainable contribution to reduce Zn deficiency in humans. The identified rice genotypes like Kumra Ghor, Ghigoj, Tilek Kuchi etc. showing Zn bio-synthetic gene would be used as breeding materials for developing rice varieties possessing optimum Zn concentration in their endosperm through MAS process.

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Conflict of interest

None to declare.

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