Asian Australas. J. Biosci. Biotechnol. 2016, 1 (1), 1-14

Asian-Australasian Journal of Bioscience and Biotechnology
ISSN 2414-1283 (Print) 2414-6293 (Online)
www.ebupress.com/journal/aajbb

Article

DNA fingerprinting and chemical analysis of rice genotypes for iron content

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Received: 03 March 2016/Accepted: 04 April 2016/ Published: 30 April 2016

Abstract: Iron deficiency causes anemia in human body. So identification of iron rich rice genotypes as well as biofortification of staple food crops is an effective way to overcome such malnutrition problems. A total of 46 local rice landraces of Bangladesh were used in chemical analysis and DNA fingerprinting to study their ability to synthesize and accumulate iron content in the grain. Rice plants were grown and their grains were collected and digested by acidKNO_3:$HClO_4$. iron concentrations were measured from The highest iron content was found Kumra Ghor (168.52ppm) and the lowest in Patnai Balam (0.45 ppm). The SSR markers were used to determine the allelic diversity and relationship among the selected rice germplasms for iron content. Out of 10 SSR primers, 5 primers showed DNA amplification and polymorphism among all the genotypes. Variation was found in number of alleles, allele frequency, Polymorphism Information Content (PIC) and gene diversity. The primer, RM35 having motif (GA) 19 also yielded the highest number of alleles (23) and highest PIC value (0.946). A total of 72 bands were recorded by using 5 SSR primers in all genotypes. The number of alleles per locus ranged from 4 to 23 with an average of 14.40 out of 72 bands. The UPGMA Dendrogram based on Nei's (1973) genetic distance placed the varieties into 6 distinct clusters. Most of the primers showed the highest Polymorphism Information Content (PIC). Based on this study, the larger range of similarity values using SSR markers provides greater confidence for the assessment of genetic relationships among the varieties. The information obtained from chemical analysis and SSR profiling helps to identify the varieties containing iron content. Genotypes with high iron content could be used as breeding materials to develop nutrient rich rice varieties in order to combat iron deficient problems in population of Bangladesh.

Keywords: bio-fortification; gene diversity; PCR; rice landrace; rice microsatellite

1. Introduction

Rice is a predominant staple food and a major source of dietary carbohydrate or energy for more than half of the world's population. Poor grain micronutrient contents (Iron, Zinc and Pro-vitamin A) in cereals are the primary cause of prevalent nutritional deficiency amongst population having cereals based diet living in the developing world. Although, rice is not a major source of mineral in the diet, any increase in its mineral concentration could significantly help to reduce iron deficiency. Identification as well as development of rice varieties with higher amount of micronutrients is an important way to overcome these malnutritions. Understanding of the genetic basis of iron accumulation in grain and plant is the prerequisite for manipulation of these micronutrients. The rapid development of molecular technology provides great opportunities to enhance nutritive values of traditionally cultivated crops. Bio-fortification of staple food crops for enhanced micronutrient content through

genetic manipulation is the best option available to alleviate hidden hunger with little recurring costs. Growing of iron bio-fortified rice is only the feasible way of reaching the iron nutrient to the malnourished population in rural Bangladesh. In addition to agronomical management, selection of genotypes with high efficiency of iron accumulation in the endosperm and their bioavailability from existing germplasm collection may be an efficient and reliable way to deliver iron nutrition benefits to farmers and local population. Some wild relatives of rice were found to have higher grain iron concentrations compared with the cultivated rice germplasm. These can be used as breeding materials in biofortification system. 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) screened 113 rice landraces from 12 provinces of China and reported that japonica rice had higher iron than that of *Indica* rice variety. Belarra *et al.* (1996) evaluated 11,400 rice samples of brown and milled rice iron and zinc content during 2006-2010. They found that brown rice had 10-11 ppm iron and 20-25 ppm zinc while milled rice had 2-3 ppm iron and 16-17 ppm Zn. Banerjee *et al.* (2010) screened 46 rice lines including cultivated and wild accessions and showed that wild rice accessions have higher grain iron and zinc concentration. Micronutrient-dense cultivars can be selected from within existing rice germplasm, or can be generated de novo through genetic modification. Plant breeders may be involved in breeding staple food crops with more iron need to identify donor parents carrying the target traits. By increasing the iron content of food staples through plant breeding and marker assisted selection (MAS), the entire iron status distribution curve can be shifted to that smaller group of nutrient deficient persons in the world. Hence, agriculture must now focus on a new paradigm that will not only produce more food, but also bring us better quality food as well.

2. Materials and Methods

2.1. Experimental site, duration and selected genotypes

Seeds of the 46 local rice varieties (Table 1) with diverse genetic background were collected from the Gene Bank of BINA, Mymensingh. All accessions were grown in the molecular lab and experimental field of Biotechnology and Plant Breeding division of BINA. After collection of 21 day young leaves for DNA samples, seedlings were transplanted into main field. After harvesting, seeds were collected from each of the genotypes for further chemical analysis in Agricultural Chemistry Lab. of BAU and soil science division of BINA during December, 2013-February, 2014. The molecular characterizations were done using leaf sample in the Genetic Fingerprinting Laboratory of BINA, BAU, Mymensingh-2202, during 5 August, 2013 to 30 April, 2014.

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

After incubation into oven at 54°C for 48 hours, 5-7 seeds were germinated in the wet blotting paper in petridishes followed by transplanting into small plastic tray. After 21-25 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 polythene bag with proper labeling separately for further isolation of genomic DNA. Thirty two days older 2-3 seedlings per hill were transplanted to the main field of Biotechnology Division at BINA. Harvesting was done depending upon the maturity of different genotypes. The date of harvesting was confined when 90% of the grain attained golden yellow color. Harvesting was completed by 10-20 December, 2013. After harvesting, (200-220g) seeds were collected from each of the genotypes for further chemical analysis.

2.3. Chemical analysis

The chemical analysis was done in a Completely Randomized Design (CRD) for iron content maintaining two replications for each genotype.

2.4. Processing of harvested seeds for chemical digestion

At first, the collected rice seeds were oven dried at 55^0C temperature for 48 hours to facilitate the de-husking process. The oven dried seeds were then placed in winnower and rubbed across it followed by de-husking carefully. The de-husked seeds were then ground into powder separately with the help of a mechanical grinder. The rice grain powder of each genotypes was then stored very carefully in a polythene bag with proper labeling.

2.5. Determination of iron content from rice grain sample

Iron (Fe) content of rice grain samples was determined by using Atomic Absorbance Spectrophotometer (AAS) as stated by Lindsay and Novell (1978). In this process the samples were digested by di-acid mixture of Nitric

acid ($HNO_{3)}$ and choric acid ($HClO_4$ in 2:1 ratio (Bhatia and Khetarpaul, 2012). The detailed procedure of this process described below.

2.6. Chemical digestion of rice grain powder

From each genotype, about 1g rice grain powder was taken in 150 mL conical flask and 15 mL of di-acid mixture (HNO_3: $HClO_4$=2:1) was added to it. It was kept overnight at room temperature by covering the mouth of the conical flask with aluminum foil paper. Then the conical flask was placed on sand bath followed by heating at temperature 180~200^{0}C for 30~40 minutes. After a few minutes (25~30 min) brown fume was evolved which indicated the starting of digestion process. Finally white fume was seen by clearing the solution. Electric power supply was switched off when 2-3 mL solution was noticed at the bottom of the conical flask. Three blank solutions were also performed along with all the sample solution. After heating, the digested sample was cooled for 20 minutes. Then about 25~35 mL distilled water was added to each conical flask to avoid filtration problem. Then this solution was filtered into a 50mL volumetric flask and the volume was made up to the mark (50 mL) with distilled water. The 50 mL solution was then transferred into a plastic bottle for each genotype for the further utilization. The plastic bottle was stored at a room temperature. Total procedure is outlined as a flow diagram (Figure 1).

2.7. Standardization of AAS and estimation of grain iron content

Normally the iron atoms remain in ground state. They absorb energy under flame condition when these are subjected to radiation which is proportional to the specific wavelength. The absorption of radiation is proportional to the iron concentration. Iron content was estimated in the aliquot of seed extract by using AAS at 248.33nm wavelengths at Soil Science Division, BINA, Mymensingh. Concentration was expressed in parts per million (ppm). For standardization, the AAS machine was setup and calibrated with seven standard iron concentrations of 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ppm with absorbance readings of -0.001, 0.045, 0.085, 0.164, 0.242, 0.300 and 0.361 respectively to estimate the iron concentration of all the rice genotypes accurately. A minimum of two replications for each sample were analyzed for iron content. The variation in replications for each sample did not exceed ± 2ppm for iron concentration. The mean of the two replicates was presented in results. The amount of iron content (ppm) =AAS concentration reading× Conversion factor (50).

2.8. Molecular analysis

2.8.1. Isolation of rice genomic DNA

The genomic DNA was extracted from 21 day young leaves using N-Cetyl-N, N, N-trimethyl ammonium bromide (CTAB) method (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 de-ionized 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^{0}C 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^{0}C in the hot water bath. About half volume (400-900µL) of PCI (Phenol: Chloroform: Iso-amylalcohol= 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 (10mM 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.

2.8.2. Confirmation, quantification and optimization of DNA concentration

The amount of genomic DNA was quantified at 260nm spectrophotometrically at PMH 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 ton 25 ng/µL for further PCR analysis.

$$\text{DNA conc. } (\mu g/\mu L) = \left[\text{Absorbance} \times \frac{\text{Vol. of deionized water } (\mu l)}{\text{Amount of DNA } (\mu l)} \times \text{conversion factor x } 1000 \right]$$

2.8.3. Identification and selection of SSR markers

A set of ten microsatellite primer pairs (RM9, RM21, RM23, RM35, RM167, RM180, RM217, RM296, RM400 and RM520) distributed in the rice genome for iron and Zinc content were identified from the available data-based search (http://www.gramene.org/) for rice SSR markers as described by McCouch *et al.* (1997), Temnykh *et al.* (2001), McCouch *et al.* (2002) and Shankar Ilango and N Sarla (2010).

2.8.4. 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 an 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.

2.8.5. Polymorphism survey

The identified 10 primers were tested against 3 randomly selected varieties with a recommended PCR thermal profile. Five primers showed good resolution for DNA banding pattern among the ten primers. Based on better responsiveness in amplifying the target genomic region of template DNA, the expected PCR product sizes in base pairs was then checked. The selected 5 primer pairs viz. RM9, RM23, RM35, RM217 and RM296 representing chromosome numbers 1, 1, 1, 6 and 9 of rice genome (Temnykh *et al*., 2001) with clear and expected amplified product sizes were selected and used for microsatellite analysis for iron content among 46 rice varieties in the present study. The details of the primers are given in Table 2.

2.8.6. Electrophoresis and visualization of PCR products

PCR was confirmed by electrophoresis on 8% polyacrylamide gel. Prior to electrophoresis each PCR-products 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 and the progress of electrophoresis and to increase the weight of the sample so that it 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 and final level of buffer was ~5mm above the gel. After loading of DNA sample into gel well carefully, a 25 bp molecular weight marker DNA or ladder was loaded on either 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 respective marker using the power supply. The DNA migrates from negative to positive electrode. The electrophoresis was stopped after the bromophenol blue dye had reached three-fourths of the gel length and then electric power supply connection was switched off. PCR-products were electrophoresed on 8% polyacrylamide gel. For documentation, the gel was carefully taken out from the electrophoresis chamber 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 was photographed by using a Gel Doc camera and finally saved in computer.

2.8.7. Scoring and analysis of microsatellite data

Microsatellite DNA profiles of all rice varieties using five primers are shown in Figure 2 (a-e). Molecular weights for microsatellite products were also estimated with AlphaEaseFC 4 software from DNA band images. 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. Estimation of Nei's genetic distance values (D) and construction of UPGMA Dendrogram was constructed using the software POWER MARKER (version 3.23) (Yeh *et al.*, 1999).

3. Results and Discussion

3.1. Estimation of iron concentration by atomic absorbance spectrophotometer (AAS)

The analyses of variance for iron concentration of selected rice genotypes are presented in Table 3. The ANOVA revealed significant variation for iron concentration due to genotypes at 0.01 level of probability.

3.2. Mean performance of 39 rice genotypes for iron concentration

The mean performances of 46 rice genotypes for iron concentration are presented in Table 4. In chemical analysis, a wide range of variation for iron concentration was found among all the rice genotypes. The analysis of variance revealed significant variation for iron concentration of the genotypes at 0.01 level of probability (Table 3). This ANOVA suggested that there were inherent genetic differences among the genotypes and this character might be effective for further crop improvement. Among 46 rice landraces, iron concentration ranged from 0.45 ppm to 168.52 ppm where maximum iron was found in Kumra Ghor (168.52ppm), medium in Kalo Mota (59.57ppm) and minimum in Patnai Balam (0.45ppm). Their on content of local landraces is higher than the others which also supported by the findings that were earlier reported by G. S. Jahan *et al.* (2013), Ilango and Sarla (2010), Banerjee *et al.* (2010) and Anuradha *et al.* (2012). Banerjee *et al.* (2010) screened 46 rice lines including cultivated and wild accessions and showed that wild rice accessions have higher grain iron concentration. Anuradha *et al.* (2012) reported that they analyzed brown rice of 126 accessions of rice genotypes for iron concentration. The iron concentration ranged from 6.2 ppm to 71.6 ppm and the local accessions had the highest Fe. In this study some local landraces showed higher iron concentration than the previously reported. Thus, local landraces would be a good source for biofortification of popular rice cultivars using different breeding approaches.

3.3. PCR amplification of all rice genotypes

Polymerase Chain Reaction was done to check each sample for parental polymorphism using specific Rice Microsatellite (RM) primers. Out of 10 primers, 5 RM primers showed polymorphism. Fifty percent polymorphism was found among the markers calculated using the following formula:

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

3.4. 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 (RM9, RM23, RM35, RM217 and RM296) amplified were found to be polymorphic. Using 5 primers across 46 varieties 72 alleles were identified. The number of alleles ranged from 4 to 23 per locus. The locus RM35 had the highest number of alleles (23) and the locus RM9 had the lowest number of alleles (4) (Table 8). Yang *et al.* (1994) found up to 25 alleles for 10 microsatellite markers among 238 accessions of *Indica* and *Japonica* cultivars and landraces.

3.5. Gene diversity

According to Nei's (1973), the highest level of gene diversity value (0.948) was observed in loci RM35 and the lowest level of gene diversity value (0.563) was observed in loci RM9 with a mean diversity of 0.834 (Table 8). 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.

3.6. Major allele

The allele with maximum frequency is known as major allele. RM9 showed major null allele but RM23 showed major allele frequency (0.261) and size 143bp while RM35 showed major allele with minimum frequency 0.087 and size 197bp (Table 8).

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	ChiniShail	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	PatnaiBalam	BINA Gene Bank	G29	Sali	BINA Gene Bank
G7	TilekKuchi	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	DharShail	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	KathiGoccha	BINA Gene Bank
G13	GengengBinni	BINA Gene Bank	G36	KhejurChori	BINA Gene Bank
G14	KumraGhor	BINA Gene Bank	G37	Ghocca	BINA Gene Bank
G15	Kalmilata	BINA Gene Bank	G38	AsamBinni	BINA Gene Bank
G16	HatiBajor	BINA Gene Bank	G39	MarishShail	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	BhuteShalot	BINA Gene Bank	G43	Khuchra	BINA Gene Bank
G21	BajraMuri	BINA Gene Bank	G44	ShahebKachi	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 SSR primers used for polymorphism survey.

Primer	Sequence (5'-3')	PCR Product Size (bp)	Ta (^{0}C)	Chrom.	Reference
RM9*	F=GGTGCCATTGTCGTCCTC R=ACGGCCCTCATCACCTTC	136	55 55	1	Shankar Ilango and N. Sarla (2010)
RM21	F=ACAGTATTCCGTAGGCACGG R=GCTCCATGAGGGTGGTAGAG	157	55 55	11	
RM23*	F=CATTGGAGTGGAGGCTGG R=GTCAGGCTTCTGCCATTCTC	145	55 55	1	
RM167	F=GATCCAGCGTGAGGAACACGT R=AGTCCGACCACAAGGTGCGTTGTC	128	55 55	11	
RM180	F=CTACATCGGCTTAGGTGTAGCAACACG R=ACTTGCTCTACTTGTGGTGAGGGACTG	110	55 55	7	
RM217*	F=ATCGCAGCAATGCCTCGT R=GGGTGTGAACAAAGACAC	133	55 55	6	
RM400	F=ACACCAGGCTACCCAAACTC R=CGGAGAGATCTGACATGTGG	321	55 55	6	
RM520	F=AGGAGCAAGAAAAGTTCCCC R=GCCAATGTGTGACGCAATAG	247	55 55	3	
RM35*	F=TGGTTAATCGATCGGTCGCC R=CGACGGCAGATATACACGG	207	55 55	1	Tenmykh *et al.* (2001)
RM296*	F=CACATGGCACCAACCTCC R=GCCAAGTCATTCACTACTCTGG	123	55 55	9	

Ta= Annealing Temperature, *Primers showing effective polymorphism used for SSR analysis.

Table 3. Analysis of variance for grain iron concentration of 46 rice genotypes.

Source of variation	d.f.	S.S	M.S	Calculated F-value	Tabulated F Value	
					0.05	0.01
Genotype	45	107224.576	2382.763**	6665.04	1.646	2.035
Error	46	15.853	0.347			
Total (Corr.)	91	107240.429				

** indicates significant at 0.01 probability

Table 4. Mean performance of 46 rice genotypes based on grain iron (Fe) concentration.

SL. No.	Variety	Iron Conc. (ppm)	SL. No.	Variety	Iron Conc. (ppm)
G1	Rupessor	5.13	G28	Lal40	2.98
G2	Karengal	25.20	G29	Sali	3.30
G3	Kalomota	59.57	G30	Gotamala	4.12
G4	Nona Kochi	35.38	G31	Rani Shalot	0.55
G5	Vashiara	1.38	G32	DharShail	9.17
G6	PatnaiBalam	0.45	G33	Malagoti	5.10
G7	TilekKuchi	112.92	G34	Volanath	5.82
G8	Bashful Balam	28.95	G35	KathiGoccha	15.33
G9	Chap Shail	23.05	G36	KhejurChori	6.95
G10	Mohime	16.8	G37	Ghocca	2.15
G11	Mowbinni	22.20	G38	AsamBinni	1.70
G12	Lalanamia	14.53	G39	MarishShail	9.78
G13	GengengBinni	14.27	G40	Raja Shail	1.75
G14	KumraGhor	168.52	G41	Nunnia	2.77
G15	Kalmilata	8.17	G42	Bogi	6.18
G16	HatiBajor	31.05	G43	Khuchra	2.82
G17	Ponkhiraj	16.10	G44	ShahebKachi	5.80
G18	Ghigoj	138.42	G45	Golapi	11.65
G19	Hogla	21.34	G46	Sadagotal	10.67
G20	BhuteShalot	15.22	CV (%)		2.99
G21	BajraMuri	2.98			
G22	Mondessor	13.88	Maximum		168.52
G23	Jolkumri	7.70	Minimum		0.45
G24	ChiniShail	8.78	Mean		19.60
G25	Pengek	3.20	$Lsd_{(0.05)}$		1.189
G26	Gota	2.33	Level of Significance		**
G27	Kherail	3.78			

Table 5. Thermal profile for PCR reaction.

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 6. Number of allele of different sizes with frequency, variance, standard deviation, 2.5% l.b and 97.5% u.b found at 5 SSR loci across 46 rice varieties.

RM9							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	28	0.609	0.005	0.072	0.500	0.739	152	2	0.022	0.000	0.022	0.000	0.065
142	20	0.217	0.004	0.061	0.109	0.326	156	4	0.043	0.001	0.030	0.000	0.109
143	12	0.130	0.002	0.050	0.065	0.217	162	2	0.022	0.000	0.022	0.000	0.065
144	4	0.043	0.001	0.030	0.000	0.087	164	4	0.043	0.001	0.030	0.000	0.130
RM23							167	2	0.022	0.000	0.022	0.000	0.065
0	1	0.022	0.000	0.022	0.000	0.043	170	6	0.065	0.001	0.036	0.000	0.130
122	2	0.022	0.000	0.022	0.000	0.065	172	2	0.022	0.000	0.022	0.000	0.065
123	8	0.087	0.002	0.042	0.022	0.174	173	2	0.022	0.000	0.022	0.000	0.087
135	2	0.022	0.000	0.022	0.000	0.065	174	2	0.022	0.000	0.022	0.000	0.043
139	2	0.022	0.000	0.022	0.000	0.043	181	4	0.043	0.001	0.030	0.000	0.087
140	4	0.043	0.001	0.030	0.000	0.109	182	6	0.065	0.001	0.036	0.000	0.152
141	2	0.022	0.000	0.022	0.000	0.065	184	6	0.065	0.001	0.036	0.022	0.130
142	14	0.152	0.003	0.053	0.065	0.283	185	4	0.043	0.001	0.030	0.000	0.109
143	24	0.261	0.004	0.065	0.152	0.391	186	4	0.043	0.001	0.030	0.000	0.109
144	10	0.109	0.002	0.046	0.022	0.196	187	6	0.065	0.001	0.036	0.000	0.130
145	18	0.196	0.003	0.058	0.087	0.326	188	6	0.065	0.001	0.036	0.000	0.152
146	2	0.022	0.000	0.022	0.000	0.065	190	4	0.043	0.001	0.030	0.000	0.109
147	2	0.022	0.000	0.022	0.000	0.065	193	4	0.043	0.001	0.030	0.000	0.109
RM217							197	8	0.087	0.002	0.042	0.022	0.152
0	8	0.174	0.003	0.056	0.087	0.261	200	6	0.065	0.001	0.036	0.022	0.130
90	2	0.022	0.000	0.022	0.000	0.065	201	4	0.043	0.001	0.030	0.000	0.109
91	8	0.087	0.002	0.042	0.022	0.152	204	2	0.022	0.000	0.022	0.000	0.065
92	12	0.130	0.002	0.050	0.043	0.239	207	2	0.022	0.000	0.022	0.000	0.065
94	6	0.065	0.001	0.036	0.000	0.130	**RM296**						
95	2	0.022	0.000	0.022	0.000	0.065	0	5	0.109	0.002	0.046	0.043	0.174
96	4	0.043	0.001	0.030	0.000	0.109	114	6	0.065	0.001	0.036	0.022	0.152
99	2	0.022	0.000	0.022	0.000	0.065	115	6	0.065	0.001	0.036	0.000	0.152
110	4	0.043	0.001	0.030	0.000	0.130	116	6	0.065	0.001	0.036	0.000	0.130
112	2	0.022	0.000	0.022	0.000	0.065	117	6	0.065	0.001	0.036	0.000	0.130
115	2	0.022	0.000	0.022	0.000	0.065	118	12	0.130	0.002	0.050	0.065	0.217
116	2	0.022	0.000	0.022	0.000	0.065	119	4	0.043	0.001	0.030	0.000	0.087
117	6	0.065	0.001	0.036	0.000	0.130	120	6	0.065	0.001	0.036	0.022	0.130
124	2	0.022	0.000	0.022	0.000	0.043	121	2	0.022	0.000	0.022	0.000	0.087
126	2	0.022	0.000	0.022	0.000	0.065	122	4	0.043	0.001	0.030	0.000	0.109
129	4	0.043	0.001	0.030	0.000	0.109	123	6	0.065	0.001	0.036	0.000	0.130
133	4	0.043	0.001	0.030	0.000	0.109	124	16	0.174	0.003	0.056	0.087	0.261
135	6	0.065	0.001	0.036	0.022	0.152	125	8	0.087	0.002	0.042	0.000	0.152
136	6	0.065	0.001	0.036	0.000	0.130							

Table 7. Molecular analyses results found in all varieties for 5 SSR markers.

Marker	Repeat motif	Sample size	No. of observation	Allele size ranges(bp)	Diff. (bp)	Hetero zygosity	Availability	f
RM9	(GA)15GT(GA)2	46.0	46.0	142-144	2	0.00	1.00	1.00
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
RM296	(GA)10	46.0	46.0	114-125	11	0.00	1.00	1.00
Mean	------	46.0	46.0	---------	42	0.00	1.00	1.00

Table 8.Size and frequency of alleles and diversity index at 5 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. (%)							
RM9	0.00	0.609	4.00	4.00	1.00	28.0	0.512	0.563	0.00
RM23	143	0.261	13.00	13.00	8.00	1.00	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	8.00	0.911	0.917	0.00
RM296	124	0.174	13.00	13.00	3.00	5.0	0.896	0.904	0.00
Total	464	1.305	72.00	72.00	40.0	42.00	4.094	4.178	0.00
Mean	92.80	0.261	14.40	14.40	8.00	8.40	0.819	0.834	0.00

Table 9. Categorization of 46 rice genotypesintosix clusters based genetic distance and UPGMA dendrogram.

Clusters	Genotypes found	Percent (%)	Genotypes
I	9	19.565	G32, G36, G40, G21, G22, G35, G37, G41, G27
II	4	8.695	G1, G33, G13, G44
III	2	4.347	G12, G43
IV	3	6.521	G5, G8, G7
V	3	6.521	G34, G42, G45
VI	25	54.347	G6, G24, G4, G15, G2, G3, G10, G11, G23, G31, G26, G28, G20, G29, G30, G9, G14, G18, G39, G17, G25, G16, G19, G38, G46
Total	46	100	46 Rice genotypes

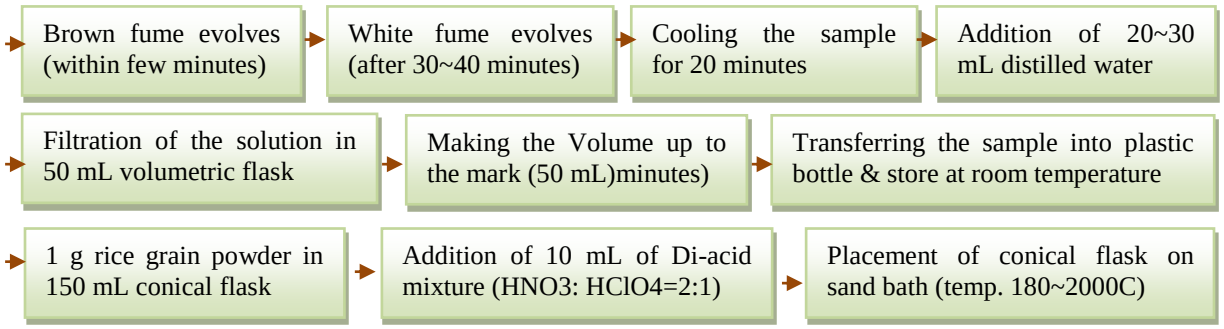

Figure 1. Digestion of rice grain powder by di-acid mixture (HNO_3: $HClO_4$=2:1) to estimate grain iron content.

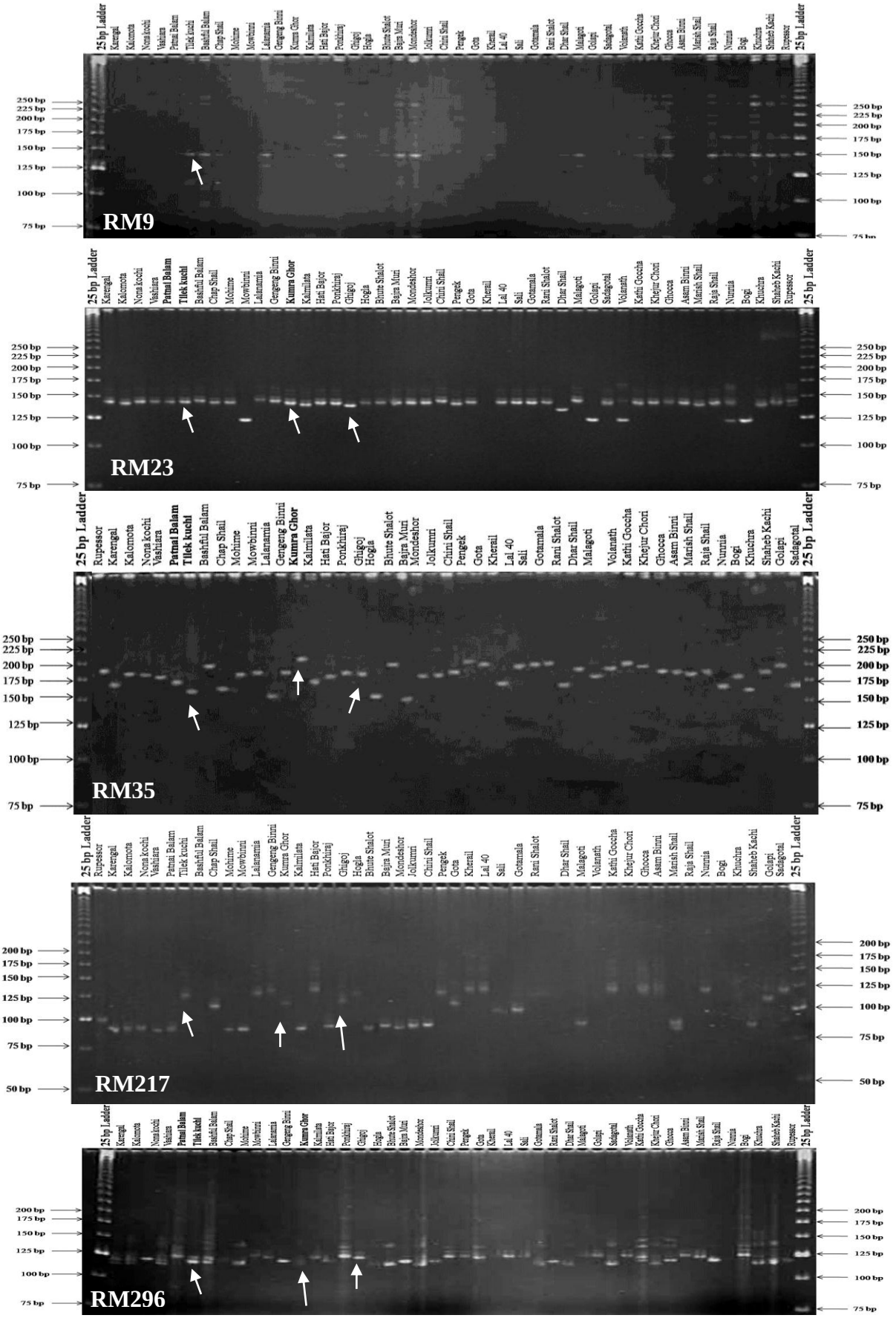

Figure 2. Microsatellite SSR DNA profiles of 46 rice genotypes for iron content using RM9, RM23, RM35, RM217 and RM296 where each consecutive lane represents a single variety. Each arrow indicates the DNA Band position for iron rich varieties (Kumra Ghor, Ghigoj, Tilek Kuchi).

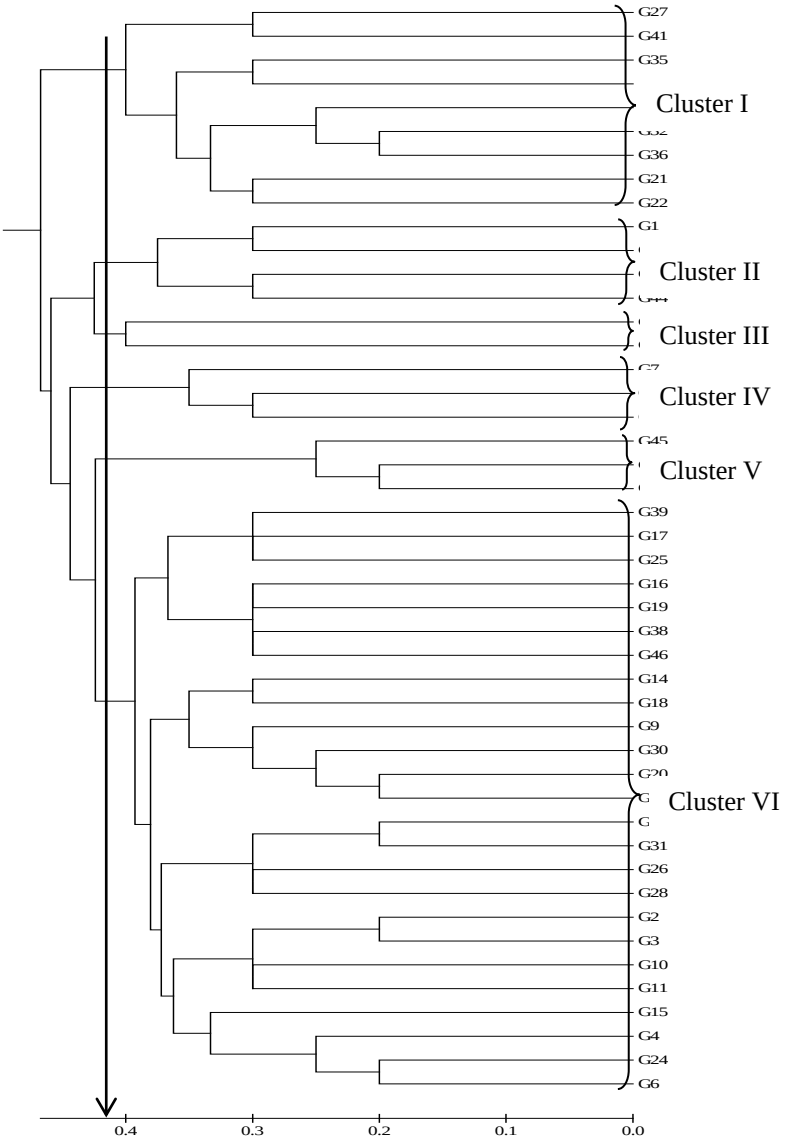

Figure 3. UPGMA Dendrogram based on Nei's (1973) genetic distance, summarizing data on differentiation. The dendrogram was cut at 50% of average genetic distance (0.417) to separate sub-clusters. Genotype's serials were mentioned in the Table 1.

3.7. Rare allele

The allele with frequency less than 0.05 is known as rare allele. Maximum rare alleles (16) were found for RM35 followed by RM217 (12) and minimum (1.0) was found for RM9 with an average of 8.0 rare alleles across 46 rice genotypes for 5SSR markers (Table 8).

3.8. Null allele

Null allele means no appearance of allelic band against the genotype for the selected marker. Maximum null alleles (28) were found for RM9, there is no null allele for RM35 among 46 rice genotypes with an average of 8.40 (Table 8).

3.9. Allelic diversity

All the information of allele size, number, frequency, variance, standard deviation, % right border and % upright border were mentioned in Table 6. Using 5 primers across 46 rice varieties, a total of 72 alleles were found in the present study where RM35 showed highest number of observed number of alleles (23) followed by RM217 (19), RM296& RM 296 (13) and RM9 (4) in descending order (Table 8). 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, RM9 presented the smallest allele size range (2bp), while RM35 presented the largest allele size range (55bp) (Table 7).

3.10. 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. The markers in the specific chromosome and the allele size along with their frequencies and PIC values have been shown in the Table 8. The PIC values for 5 primers obtained in the present study varied from 0.512 for RM9 to 0.946 for RM35, with an average PIC value of 0.819 (Table 8). Among the markers used in this study RM35 showed higher PIC values and RM9 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*., 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*. (2001) 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 (Table 8).

3.11. Banding pattern of 46 rice genotypes using 5 SSR markers

All the figures of banding patterns of 46 rice genotypes for molecular analysis of iron content using five polymorphic SSR primers are presented in Figure 2 (a-e). In this experiment, five polymorphic SSR markers *viz.*, RM9, RM23, RM35, RM217 and RM296 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 iron content.

3.12. Genetic distance

The values of pair-wise comparisons of Nei's (1973) genetic distance (D) between genotypes were computed from combined data for the 5 primers, ranged from 0.00 to 1.00. Higher genetic distance was observed between some genotypes pair than the other genotypic combination. The means of genetic distances between lines were used to evaluate the genetic diversity of different lines. Highly diversified genotypes could be useful in breeding program to have potential genetic gains.

3.1.3. UPGMA Dendrogram or Phylogenic tree

Forty six lines of the experiment were used to make Dendrogram based on Nei's (1973) genetic distance using UPGMA Dendrogram. In this study, 46 rice lines have been differentiated into six main clusters. The clusters were separated into several sub-clusters based the 50% of average genetic distance (0.417). The Dendrogram showed that the lines were closely related belonging to the same cluster were not genetically diverse while the lines belonging to different cluster were genetically diverse in origin.

3.14. Cluster analysis

Cluster analysis was performed using the UPGMA method to group the studied genotypes based on similarity coefficient. Mainly six clusters were formed at genetic similarity level of 0.10-0.467 (Figure 3). Cluster I, II, III, IV, V and VI contained 9, 4, 2, 3, 3 and 25 genotypes with percentage of 19.565, 8.695, 4.347, 6.521, 6.521 and 54.347, respectively (Table 9). Among the total genotypes those which showed comparatively same banding patterns were grouped into same cluster. These clusters were divided into sub clusters in many folds.

4. Conclusions

Plant breeding and biotechnology tools are good for fighting micronutrient malnutrition. The result of the present work represents the first approach in understanding the biofortification of micronutrient (Fe) in Cereals like Rice. Plant-breeding with MAS holds great promise for making a significant, low-cost, and sustainable contribution to reducing deficiencies of micronutrients, particularly of minerals, in humans and may have important spin-off effects in increasing farm productivity in developing countries in an environmentally beneficial way. Molecular marker technology expedites the development of rice varieties with improved iron content through identified genomic regions. The iron content in brown and milled rice of national and

international germplasm need to be estimated for identification of donors for future deployment in the nutritional breeding program and also to get mapping information on association of iron content in grains. Rice lines in the genetic background of elite rice varieties possessing optimum iron concentration in the endosperm will be developed and released for cultivation. This strategy holds great promise for significantly reducing recurrent expenditures required for these higher-cost, short-run programmes by significantly reducing the numbers of people requiring treatment. Because staple foods are eaten in large quantities every day by the malnourished poor, the delivery of enriched staple foods can rely on existing consumer behaviors. So, the rice landraces with high grain iron concentration can be used as breeding materials to develop nutrient rich rice varieties consumed as staple food especially for the poor and malnourished people living in Bangladesh.

Acknowledgements
The principal author is thankful to Bangladesh Agricultural Research Council (BARC), Ministry of Science Technology (MOST) for their financial support and Department of Genetics and Plant Breeding, Bangladesh Agricultural University; Plant Breeding and Biotechnology Division of Bangladesh Institute of Nuclear Agriculture (BINA) for technical assistance.

Conflict of interest
None to declare.

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