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MOLECULAR CHARACTERIZATION OF PARENTAL LINES OF RICE AIMING TO ADDRESS HIGH YIELD AND NUTRITIONAL QUALITY UNDER DROUGHT AND COLD STRESS CONDITION

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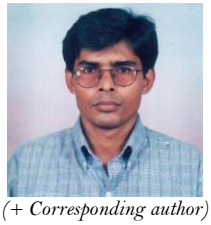

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ABSTRACT

Abiotic stresses limit crop growth at different growth stages resulting low yield of rice. Molecular characterization of parental materials gives precise information on the extent of genetic diversity exists between them. A set of 60 SSRs randomly distributed over 12 chromosomes were used to analyze eight cultivars intend to be used as parent in breeding programs to address cold and drought tolerance, and nutritional quality of rice. A total of 300 alleles were detected across the cultivars for 51 polymophic markers with 5.88 alleles per loci. On average 30.6% of the genotypes shared a common allele at any given locus. UPGMA cluster analysis showed that 67% common alleles were shared by the cultivars. The cultivars were clearly grouped into two distinct clusters at 67.0% genetic similarity. Hbj. BVI showing high tolerance to cold stress at seedling stage differed from cold susceptible BR1, BRRI dhan28 and BRRI dhan29 by 33% alleles while BR18 that showed moderately cold tolerance differed by 29.0 - 29.9% alleles which indicated that only 3-4% alleles difference caused higher cold tolerance in Hbj. BVI. The moderate genetic distance between cold tolerant Hbj. BVI and high yielding BRRI dhan28 and BRRI dhan29 indicated that there is higher possibility of obtaining high yielding cold tolerant segregants from the crosses between them. On the other hand, Kalobokri, which had 31.7 mg zinc a kilogram of polished rice differed by 33.0% alleles from the drought tolerant rainfed low varieties BRRI dhan56 and BRRI dhan57 having moderate level of zinc (~20 mg/kg), which also indicated that crosses between them might produce progenies with higher nutritional quality under drought environment.

Contribution/ Originality: This study is one of the few studies which have investigated the genetic distance between the parental lines that are intended to be used in the breeding program to address abiotic stresses, like drought and cold tolerance with high yield potential and enhanced nutritional quality, particularly zinc content in rice.

1. INTRODUCTION

Rice (*Oryza sativa L.*) production in Bangladesh is affected by various abiotic stresses. The rainfed lowland rice is greatly affected by drought stress accounting for 46%, 37% and 73% yield loss if it occurs during flowering, maturity and both flowering and maturity, respectively [1]. On the other hand, Boro rice is suffered from critical

low temperature at different stages of growth from germination to maturity that in turn results into low yield. However, low temperature stress at booting stage produces sterile spikelets causing direct yield loss of short duration varieties up to 100% in some years in the low lying haor areas of Bangladesh. Approximately 22.4% and 20% of total rice areas in the country are affected by moderate to severe level of drought and cold stress, respectively. Landraces play very important role as genetic resource for genetic improvement of rice [2] particularly to address abiotic stresses like drought and cold. Modern plant breeding techniques can effectively use landraces having different economic traits including enhanced resistance to certain stress, better grain and nutritional quality and of course the yield contributing traits. Knowledge on genetic divergence between parents is necessary to design a breeding program in order to have transgressive segregation. Genetic diversity determines the inherent potential of a cross for heterosis and frequency of desirable recombinants in advanced generations. Several workers have emphasized the importance of genetic divergence for the selection of desirable parents [3, 4].

The estimation of genetic diversity between different genotypes is the first and foremost process in plant breeding [5]. Among various techniques available for assessing genetic variability and relatedness among crop germplasm, DNA based markers provide very effective and reliable means for measuring genetic diversity and studying evolutionary relationships. Molecular markers can reveal abundant difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management avoiding environmental influence in contrast to morphological traits. Simple sequence repeat (SSR) markers which are abundant in rice genome and cost effective in discriminating small number of varieties/germplasm have been effectively used to identify genetic variation among rice cultivars [3, 6-8]. In this study, we used eight rice varieties/cultivars to dissect genetic variation among them using SSR markers to measure the extent of genotypic differences, genetic relationship and to assist in broadening the germplasm base of future drought and cold tolerance rice breeding programs.

2. MATERIALS AND METHODS

A total of eight rice cultivars having diverged trait benefits, like drought tolerance, cold tolerance, enhanced grain zinc content, short duration and high yielding (Table 1) were analyzed using 60 SSR markers randomly distributed over the rice genome. Among the eight cultivars, BR1, BR18, BRRI dhan28, BRRI dhan29 and Hbj.B.VI were evaluated against artificial cold treatment for seedling stage cold tolerance following protocol described in Khatun, et al. [9]. Cold tolerance was estimated based on leaf discoloration (LD) score following SES of IRRI, % Survival plants at day of scoring and % Recovery of plants after seven days of cold stress withdrawal. BRRI dhan56 and BRRI dhan57, two drought tolerant varieties showing no significant yield losses even when ground water table remained 70-80 cm below the surface at reproductive stage [10] were included in this study. All the genotypes except Hbj.BVI were analyzed for polished grain zinc content using X-Ray Fluorescence method in X-Supreme 8000.

For SSR analysis, DNA was extracted from young and actively growing fresh leaves using miniprep modified CTAB method as described by Virk, et al. [11]. Polymerase chain reaction (PCR) was performed in 10µl volume containing 2µl of genomic DNA, 5.3µl of DDH_2O, 1µl of 1X PCR buffer, 1µl of 0.1mM dNTP mix, 0.5µl of 0.25uM of each primer, 2µl of Taq polymerase of 1U. The temperature cycles were programmed at 94°C for 5 min(initial denaturation), 94°C for 30 sec (denaturation), 55°C for 30sec (primer annealing), 72°C for 60sec (extension), 72°C for 5 min (final extension) and 10°C forever (storage). The PCR products were detected using 6% polyacrylamide gel electrophoresis. DNA bands were visualized in a UV transilluminator with ethidium bromide staining. Molecular weight of clearly resolved and unambiguous band was determined comparing with the known sized marker DNA using Alpha Ease FC 4.0. Polymorphic Information Content (PIC) values were calculated for each SSR loci using Power Marker V3.25 [12] based on the formula developed by Anderson, et al. [13].

$$PICi = 1 - \sum_{j=1}^{n} P^2_{ij}$$

where, Pij is the frequency of the jth allele for the ith marker and is summed over n alleles.

The similarity matrices deduced from simple matching coefficient in PowerMarker analysis were subjected to unweighed pair-group arithmetic average (UPGMA) clustering analysis and represented in dendrogram form using NTSYS-pc program [14].

3. RESULTS

3.1. Evaluation for Seedling Stage Cold Tolerance

All the genotypes varied significantly in all three cold related traits. LD scores ranged from 2.0 to 8.7 among the genotypes. The highest LD was obtained with BR1 (8.7) followed by BRRI dhan28 (7.3) and BRRI dhan29 (7.0), while the lowest LD was observed with Hbj.BVI followed by BR18. Per cent survivability and % recovery values were also highest with Hbj.BVI followed by BR18, while BR1 had the lowest values for these traits. BR18 and Hbj.BVI were significantly different from the rest of the three genotypes in LD score. In % survivability and % recovery BR18 and Hbj.B.VI were also significantly different from other three genotypes and they were significantly different from each other as well (Table 2).

3.2. Evaluation of Polished Grain Zinc Content

The genotypes showed wide variation in grain zinc content ranging from 14.5 to 31.7 mg/kg. The highest zinc content was observed with the landrace Kalobokri, while all the cultivated varieties had zinc value close to 15 mg/kg except BRRI dha57, which had 20.8 mg zinc a kilogram of polished rice grain (Table 3). BRRI dhan28 and BRRI dhan29 had 15.6 and 16.9 mg zinc in a kilogram of polished rice, respectively.

3.3. Polymorphic Information Content and SSR Diversity

Sixty SSR markers were analyzed for assessing genetic divergence among eight rice cultivars. Among them, seven SSRs did not amplify at all and two markers showed monomorphism. Table 4 summarizes the results obtained from the analysis of 51 SSR loci across the test cultivars. Polymorphic information content which evident the extent of polymorphism among the cultivars varied from 0.511 to 0.861 with an average of 0.758. The highest PIC value was observed with RM 6024 and the lowest with RM193, RM 7193, RM335 and RM 1282. Out of 51 markers, 7 markers on chromosome 1, 6 markers on each of chromosome 2 and 6, 5 markers on each of chromosome 3 and 7, 4 markers on each of chromosome 12, 3 markers on each of chromosome 4, 5 and 11 and, 2 markers on each of chromosome 8, 9 and 10 were found polymorphic. A total of 300 alleles across the cultivars were detected at 51 SSR loci. The average number of alleles per locus was 5.88 with a range from 3 (RM6024 and RM6370) to 8 (RM193, RM335, RM1282 andRM7193). Among 51 SSRs, 3 alleles were detected for 2 markers, 4 alleles for 5 markers, 5 alleles for 7 markers, 6 alleles for 24 markers, 7 alleles for 9 markers and 8 alleles for 4 markers. The frequency of occurrence of an allele at each locus ranged from 12.5 % (RM193, RM335, RM1282 andRM7193) to 50% (RM329, RM497, RM6023, RM6024, RM6370 and RM7341) with a mean of 30.6%. The occurrence of number of alleles per loci was found variable irrespective of the numbers of repeat motifs and their base composition. Among 51 SSRs, 26 makers anchoring dinucleotide motif produced 154 alleles, 15 markers anchoring trinucleotide produced 82 alleles, 5 markers having tetranucleotide motifs produced 32 alleles and mixture of di- and trinucleotide motifs produced 32 alleles.

The UPGMA cluster analysis based on Shanon indices obtained from binary data that were deduced from DNA profiles of the test genotypes for the SSR markers showed high genetic variation among the cultivars with similarity coefficient values ranging from 0.67 to 0.73 (Figure 1). The cultivars were clearly grouped into two

distinct clusters at 67.0 % genetic similarity. Local variety, Kalobokri and local improved variety, Hbj.BVI were grouped into cluster I, while BRRI varieties constellated into cluster II, which was further subdivided into two sub-clusters at 70.5% similarity. BR1 and BRRI dhan56 were grouped into one sub-cluster and rest all the varieties into other sub-cluster. BRRI dhan28 and BRRI dhan29 shared 73% common alleles between them, while both of them had 71% common alleles with BR18.

4. DISCUSSION

Genetic diversity among the progenitors is crucial for any successful breeding program. Strong genetic diversity means diverse morphological traits and potentially valuable genetic information. Rice varieties with high level of genetic variation are extremely beneficial resources for broadening genetic base of the germplasm and therefore, play a good foundation for rice breeding [15]. The genotypes of this study are highly diverged in different traits of benefits. Some of them are very high yielding but they lack some specific traits of interest. These traits are present in other specific genotypes of this study (Table 1). Among eight genotypes in this study, BRRI dhan28 and BRRI dhan29 are the two most popular and high yielding rice varieties of Bangladesh with yield potential up to 7.5 and 9.1 t/ha, respectively [16]. Since release in 1994 these two varieties are still giving the highest average yield in the farmers field in the *Boro* rice ecosystem (November to May) [17] although there a series of new high yielding modern rice varieties have been released recently in Bangladesh but they are not being cultivated widely by the farmer due to adoption lags [18]. However, both BRRI dhan28 and BRRI dhan29 lack cold tolerance which is very important to uphold the potential yield in cold prone environment. In this present study we also observed cold susceptibility of these two varieties in different cold related traits, viz. LD score, % survivability and % recovery (Table 2). Khatun, et al. [9] also reported cold sensitivity of these two varieties. Among the five genotypes tested for seedling stage cold tolerance, BR18 and Hbj.BVI showed moderate to high tolerance to cold stress at seedling stage, respectively. Importantly, BR18 was released for cold prone low lying haor areas for it long stature and Hbj.BVI is a local improved *Boro* rice of haor areas of Bangladesh. On the other hand, Kalobokri which showed higher zinc content (Table 3) is a landrace of upland Aus ecosystem. This genotype might significantly contribute in enhancement of nutritional quality in the high yielding segregating progenies when crossed with high yielding varieties like BRRI dhan28 and BRRI dhan29. Mohiuddin [19] mapped QTLs for grain zinc content from a $F_{2:3}$ population of BRRI dhan28×Kalobokri. BRRI dhan56 and BRRI dha57, the two drought tolerant rainfed low varieties having early maturing traits also showed moderate level of zinc (~20 mg/kg) in polished grains. Thus, possibility of obtaining progenies with higher nutritional quality under drought environment would be higher from the crosses of Kalobokri with BRRI dhan56 and BRRI dhan57.

SSR markers are powerful tool for analyzing genetic variability among the germplasm accessions, particularly when they are closely related [20-22]. In this study, 60 microsatellite markers were analyzed to reveal genetic distance among eight rice varieties aiming to use in a breeding program targeting high yield and nutritional quality under drought and cold prone environments. Seven markers out of 60 did not amplify and two markers showed monomorphic amplification. The non-amplification of SSRs might be due to their japonica based sequence [23] of which complementary sequence may not be present in the indica type rice in this study. On the other hand, monomorphism of the markers reflected genetic closeness of the cultivars. In closely related cultivars, these phenomena are frequent and are reported in many previous studies [22, 24-26]. However, comparatively high average PIC value (0.758) with a range from 0.511 to 0.861 that was calculated based on 51 polymorphic makers indicated that the cultivars are much diverged. In fact, the local varieties (Kalobikri and Hbj.BVI) had diverse morphological traits and specific adaption under certain stress condition than the other cultivars (Table 1). In this study, Kalobokri showed to have 31.7±0.7 mg zinc in a kilogram of polished rice and Hbj.BVI showed strong cold tolerance at seedling stage. Although, all 51 SSRs markers had higher PIC values than 0.50 (an arbitrary value which is considered as threshold value for determining informative markers), RM193, RM7193, RM335 and

RM1282 had the highest PIC values (0.861), which indicated them to be the best markers for diversity analysis (Table 4). The level of PIC values of our study is comparatively higher than the reported PIC values in previous works [27-31].

The occurrence of alleles per SSR locus ranged from 3 (RM6024 and RM6370) to 8 (RM193, RM335, RM1282 andRM7193) alleles across 51 SSRs accounting a total of 300 alleles for eight cultivars. The average number of alleles (5.88) obtained in this study was bit higher than the mean allele values reported by Etemad, et al. [32] (3.57), Hossain, et al. [31] (3.8), Matin, et al. [27] (4.4), however it was much lower than the mean allele number reported by Yasmin, et al. [33]; Xu, et al. [34]; Jain, et al. [35]; Jayamani, et al. [36]; Zeng, et al. [37] and Prathepha [38] who reported an average of 13, 11.9, 7.8, 14.6, 7.7 and 11.85 alleles per locus using US rice genetic resources, Indian quality rice germplasm, a diverse collection of Portuguses rice, rice landraces from China and Wild rice (*Oryza rufipogon*) from Northeastern Thailand and Laos, respectively. The reason behind the detection of higher number of alleles in those studies was the wider variability among the germplasm. In our study, the wide genetic distance of Kalobokri and Hbj.BVI from other varieties might contribute a lot in occurrence of higher mean allele per SSR locus. Another cause might be the low resolution of markers density covering the whole genome and less number of germplasm used in the diversity analysis. However, on average 30.6% of the total genotypes shared at least a common major allele at any given locus ranging from 12.5% (RM335 and RM1282) to 50% (RM6024 and RM6370) common alleles at each locus. The gene diversity across the SSR loci ranged from 0.594 (RM6024) to 0.875 (RM193, RM335, RM1282 andRM7193) with an average of 0.788. This higher gene diversity indicated that there was wide range genetic variation among the genotypes and this was due to inclusion of local varieties in the study. The highest number of alleles (8) at each locus for RM193, RM335, RM1282 and RM7193 also confirmed this finding. However, detection of higher number of alleles at each locus was not found correlated with the anchoring motifs of the SSR loci. Majority of the SSR markers had dinucleotide repeat motifs (GA/AG, CT/TC and TA). Di-ncleootide repeat motifs are thought to be perfect repeat motif for discerning high level of variation among the genotypes [39]. In this study, the loci with perfect dinucleotide repeat motifs detected almost similar level of alleles per locus (on average 5.9, n = 26) to those with tri-, tetra- and mixture of compound di- and tri- and tetra nucleotide motifs.

UPGMA cluster analysis also revealed the existence of genetic distance among the genotypes using Shahon similarity index. The cultivars varied among themselves in a range between 67% and 73% in common allele sharing. The cultivars were clearly grouped into two distinct clusters at 67.0% genetic similarity. Kalobokri and Hbj.BVI which shared around 67.8% common alleles were grouped into one cluster and all the HYVs constellated into a second cluster. However, the second cluster was further grouped at 70.4% genetic similarity discriminating cultivars of Bangladesh origin from Philippines origin except BR18, which was introduced from Indonesia. Cold tolerant Hbj.BVI differed from cold susceptible varieties BR1, BRRI dhan28 and BRRI dhan29 by 33% while moderately cold tolerant variety BR18 differed by 29.0 - 29.9% alleles, which indicated that 3-4% allelic difference between Hbj.BVI and BR18 was responsible genetic factors for higher cold tolerance in Hbj.BVI. Generally, modern HYVs of rice share a relatively narrow genetic background as because they are mostly derived from common progenitors [40]. The high level of genetic similarity might be due to predominance of the HYV (6 out of 8 cultivars) in this study. The moderate level of genetic distance between Hbj.BVI and the high yielding BRRI dhan28 and BRRI dhan29 indicates that there is possibility to obtained high yielding and cold tolerant segregating progenies if crosses are made between them. On the other hand, the drought tolerant BRRI dhan56 and BRRI dhan57 differed from BRRI dhan28 and BRRI dhan29 by only 29.6% and 28.85% alleles and from Kalobokri by 33% alleles. These moderate level of allele difference might produce better recombinant if crosses are made between them, particularly to address zinc nutritional quality of rice under drought prone environments, as Kaloborkri possesses high zinc content and drought tolerant BRRI dhan56 and BRRI dhan57 have moderate level of zinc in their grains.

5. CONCLUSION

The larger range of similarity values for cultivars revealed by the SSR markers provides greater confidence for assessment of genetic diversity and relationships, which can be used in future breeding programs. With the aid of the SSR makers used in this study, beneficial traits (cold or drought tolerance and nutritional quality) from Hbj.BVI, BRRI dhan56 or Kalobokri could be combined with modern HYVs by intercrossing. Furthermore, genetic mapping, population structure or kinships could be explored by increasing marker resolution.

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Table-1. Basic features of the genotypes used in the diversity analysis.

Rice variety	Origin	Characteristics
BR1	IRRI,	Medium duration HYV for Boro ecosystem, cold susceptible, short statured plant, SB grain
BR18	Indonesia	HYV for Boro ecosystem, cold tolerant at seedling stage, tall statured plant, long growth duration, MB grain
BRRI dhan28	Bangladesh	Medium duration HYV for Boro ecosystem, cold susceptible, LS grain,
BRRI dhan29	Bangladesh	Long duration HYV for Boro ecosystem, cold susceptible, MS grain,
BRRI dhan56	IRRI	Short duration HYV for RLR, drought tolerant, MS grain,
BRRI dhan57	Bangladesh	Short duration HYV for RLR ecosystem, drought tolerant
Hbj.BVI	Bangladesh	Local improved short duration variety suitable for irrigated condition and has strong cold tolerance at both vegetative and reproductive stage, bold grain
Kalobokri	Bangladesh	Landrace of upland ecosystem, black husk color, high grain zinc content, tall statured plant, medium duration , bold grain

Note: SB, short bold; HYV, High yielding variety; MB, Medium bold; MS, Medium slender; LS, Long slender; RLR, Rainfed lowland; Boro, Irrigated dry season rice

Table-2. Cold response of five genotypes at seedling stage under artificial cold stress of 13^0C

Genotype	Leaf discoloration score	% Survivability	% Recovery
BR1	8.7a	17.0c	3.3c
BR18	4.0b	62.3b	47.9b
BRRI dhan28	7.3a	31.0c	10.7c
BRRI dhan29	7.0a	31.0c	12.7c
Hbj.B.VI	2.0b	94.9a	80.3a

Table-3. Zinc content in polished rice grain

Genotype	Grain zinc content (mg/kg)
BR1	14.5±0.6
BR18	17.3±0.9
BRRI dhan28	15.6±0.3
BRRI dhan29	16.9±0.6
BRRI dhan56	18.3±0.2
BRRI dhan57	20.8±0.3
Kalobokri	31.7±0.7

Table-4. Diversity analysis of 51 SSR markers across 8 rice cultivars

SN	Marker	Chromosome	Repeat motif	No, of allele	Major allele frequency	Gene Diversity	PIC
1	RM259	1	(CT)17	6	0.375	0.781	0.754
2	RM329	1	(CAT)7	5	0.500	0.688	0.653
3	RM431	1	(AG)16	6	0.250	0.813	0.786
4	RM495	1	(CTG)7	5	0.250	0.781	0.746
5	RM1282	1	(AG)17	8	0.125	0.875	0.861
6	RM6840	1	(TCT)17	6	0.375	0.781	0.754
7	RM7341	1	(CATT)6	5	0.500	0.688	0.653
8	RM174	2	(AGG)7(GA)10	6	0.250	0.813	0.786
9	RM207	2	(CT)25	7	0.250	0.844	0.825
10	RM497	2	(CAC)11	4	0.500	0.656	0.605
11	RM4499	2	(TA)20	5	0.250	0.781	0.746
12	RM6023	2	(CCG)8	4	0.500	0.656	0.605
13	RM7451	2	(TAAT)8	7	0.250	0.844	0.825
14	RM168	3	T15(GT)14	6	0.250	0.813	0.786
15	RM545	3	(GA)30	6	0.250	0.813	0.786
16	RM1230	3	(AG)15	7	0.250	0.844	0.825
17	RM3586	3	(GA)12	6	0.250	0.813	0.786
18	RM6349	3	(GAA)9	6	0.250	0.813	0.786
19	RM241	4	(CT)31	6	0.250	0.813	0.786
20	RM335	4	(CTT)25	8	0.125	0.875	0.861
21	RM3333	4	(CT)15	5	0.375	0.75	0.712
22	RM1248	5	(AG)15	6	0.375	0.781	0.754
23	RM3170	5	(CT)12	5	0.375	0.75	0.712
24	RM3328	5	(CT)14	6	0.250	0.813	0.786
25	RM6024	5	(CCG)8	3	0.500	0.594	0.511
26	RM30	6	(AG)9A(GA)12	7	0.250	0.844	0.825
27	RM193	6	(GCT)5	8	0.125	0.875	0.861
28	RM276	6	(AG)8A3(GA)33	7	0.250	0.844	0.825
29	RM469	6	(AG)15	6	0.250	0.813	0.786
30	RM5405	6	(TC)14	6	0.375	0.781	0.754
31	RM7193	6	(ATAG)7	8	0.125	0.875	0.861
32	RM18	7	(GA)4AA(GA)(AG)16	6	0.375	0.781	0.754
33	RM436	7	(TAA)6	6	0.375	0.781	0.754
34	RM1243	7	(AG)15	4	0.375	0.719	0.668
35	RM1362	7	(AG)25	6	0.250	0.813	0.786
36	RM6872	7	(TGG)8	5	0.375	0.75	0.712
37	RM284	8	(GA)8	6	0.250	0.813	0.786
38	RM407	8	(AG)13	6	0.375	0.781	0.754
39	RM3120	8	(CA)12	7	0.250	0.844	0.825
40	RM205	9	(CT)25	7	0.250	0.844	0.825
41	RM7048	9	(AATA)8	6	0.375	0.781	0.754
42	RM590	10	(TCT)10	7	0.250	0.844	0.825
43	RM3451	10	(CT)19	7	0.250	0.844	0.825
44	RM6370	10	(GAA)14	3	0.500	0.625	0.555
45	RM441	11	(AG)13	6	0.250	0.813	0.786
46	RM5349	11	(TC)13	4	0.375	0.719	0.668
47	RM6094	11	(CCT)13	6	0.250	0.813	0.786
48	RM1246	12	(AG)15	4	0.375	0.719	0.668
49	RM1261	12	(AG)16	6	0.250	0.813	0.786
50	RM7619	12	(TGTA)13	6	0.375	0.781	0.754
51	RM8216	12	(TAA)25	6	0.250	0.813	0.786
Total	-	-	-	5.88	0.306	0.788	0.758

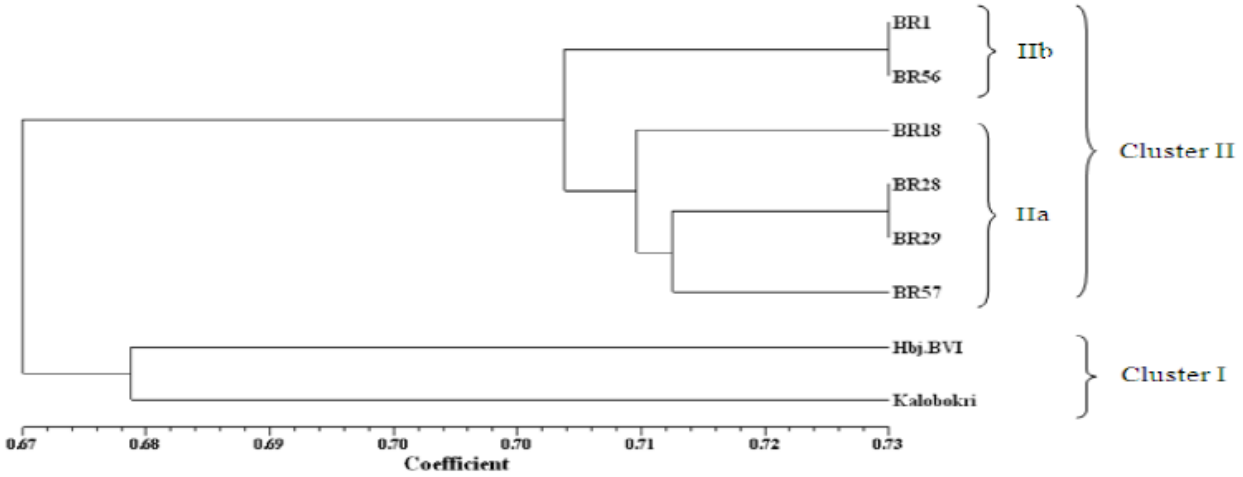

Figure-1. Dendogram derived from UPGMA cluster analysis using Shanon indices based on DNA profiling with 51 SSR markers across eight rice cultivars.