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MORPHO-MOLECULAR CHARACTERIZATION OF BANGLADESHI LOCAL BORO RICE (*Oryza Sativa* L.) GENOTYPES

T. Halder, M. E. Hoque, M. M. Islam, Liakat Ali[1], A. K. Chowdhury[2]

Bangladesh Rice research Institute
Gazipur 1701, Bangladesh.

ABSTRACT

An investigation was carried out to analysis the genetic diversity of 12 Bangladeshi local Boro rice (*Oryza sativa* L.) germplasm using morphological traits and molecular markers. Eight morphological traits (*viz.*, days to 50 percent flowering, growth duration, plant height, filled grain/panicle, 1000 grain weight and grain yield) and eight Simple Sequence Repeat (SSR) markers were used for this analysis. The plant morphological traits exhibited more variation among the genotypes tested. Several traits were found to be significantly positive in correlation coefficient analysis and thus those traits can be considered stable as demonstrated by their coefficient of variability. A set of eight SSR primer pairs was used for molecular characterization resulting 49 alleles, where average of allele number was 6.13. The polymorphic information content (PIC) values ranged from 0.67 (RM1) to 0.86 (RM314) with an average of 0.76. The highest PIC value (0.86) was obtained for RM314 which also gave maximum alleles. The PIC value revealed that RM314 was the best marker for 12 genotypes tested. The cluster analysis based on UPGMA system grouped 12 genotypes into four clusters.

Keywords: Rice genotypes; Morphological traits; Molecular marker; Genetic diversity.

INTRODUCTION

Rice is the staple food for 158.9 million people (BBS, 2016) of Bangladesh where people obtain more than 70% of their total calorie from rice. Per capita rice consumption in Bangladesh is higher than that of other country where rice is also the staple food. Two-thirds people of Bangladesh are engaged in livelihood activities related to rice. It provides nearly 43% of rural employment (BBS, 2016). Rice germplasm plays a significant role in many rice crop improvement program (Tang *et al*., 2002). Local germplasm provided "adaptability genes" for specific environmental conditions. Incorporation of adaptability genes from local germplasm may offer great potential to optimum grain yield and tolerance to different biotic and abiotic stresses. In Bangladesh, more than four thousand local rice landraces were found in different parts of the country, some of which have very nice quality, fineness, aroma, taste and protein contents (Kaul *et al.*, 1982). However, these are generally low yielding, cannot compete with modern rice varieties. Research on these local landraces was not emphasized in the past because of ever increasing demands for higher production to feed teeming millions. According to Food and Agricultural Organization (FAO, 1997), about three quarters of original

[1] Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh.
[2] Patuakhali Science and Technology University, Patuakhali, Bangladesh.

varieties of agricultural crops have already been lost from the farm fields between 1950 and 1995. It is high time to emphasize on local rice germplasm for future rice crop improvement program. Therefore, collection, characterization and conservation of traditional rice landraces are vital.

Generally rice genotypes are recognized and identified based on morpho-biochemical traits. Majority of the traits are quantitative in nature and therefore it misguides the plant scientist to recognize a particular genotypes and it is often difficult to use their criteria. Therefore, recently advent molecular characterization along with morphological traits would be the best solution. In contrast to morphological traits, molecular markers can reveal abundant difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, screening and evaluation. Among various PCR based markers, Microsatellite markers based on simple sequence repeats (SSR) have been widely used for rice. These markers are class of repetitive DNA sequences usually 2.6 bp that are distributed throughout the whole genome and are flanked by highly conserved region (Chambers and Avoy, 2000). However, these markers can detect simple sequence length polymorphism (SSLP) and are quickly relocating restriction fragment length polymorphisms (RFLPs) for many kinds of genetic approaches, largely because of their technique is easy, it just need the small amount of starting DNA, rapid turn-around time, the comparatively low cost for the users and high power of genetic resolution.

Characterization as well as diversity analysis of germplasm will provide the information to plant breeder for helping them selecting the parent for hybridization in varietal improvement program. Therefore, the objective of the study is to characterize and to evaluate the genetic diversity of 12 Bangladeshi local Boro rice germplasms using morphological traits and molecular markers.

MATERIALS AND METHODS

Twelve local Boro rice genotypes were used as experimental materials in this study. The germplasm were collected from Bangladesh Rice Research Institute (BRRI) gene bank. The accessions were grown in the field during the Boro season of 2012-13 in randomized blocks design with three replications. The accessions were grown in the field during the Boro season of 2012-13 in randomized blocks design with three replications. Thirty five days old seedling were transplanted in 3m × 3m plot with 20cm × 20cm spacing. The experiment was subjected to standard agronomic management includes aspects regarding plant geometry, fertilizer application and insect management. (BRRI Adhunik Dhaner Chash, 2012).

Evaluation of morphological traits

Data were recorded on five competitive plants per genotype from the middle row for yield and yield components such as days to 50% flowering (DFL), growth duration (GD), plant height (PLH), number of fertile tiller per hill (NFT), panicle length (PNL), filled grain per panicle (FGN), 1000-grain weight (TGW), and yield (YLD). Days to 50% flowering and total growth duration were measured from the date of seed germination to 50 % plant were heading at stage and 80% grain at hard dough stage of each plot respectively. Plant height was the distance from the ground level to the tip of the tallest panicle. NFT was counted from the number of fertile tillers per plant when single plants have been used. Grain yield (t ha^{-1}) was estimated from harvests from sampling units of $5m^2$ within the middle rows in each plot. Statistical analysis was done for quantitative traits by using CROPSTAT 7.2 and SPSS 11.5.

Molecular analysis

Leaves were collected at seedling stage (15 days) to extract the genomic DNA. Extractions of genomic DNA was carried out following miniprep DNA extraction protocol, which did not require liquid nitrogen and required only a small amount of tissue samples (Zheng *et al.,* 1995). The quality of DNA was also checked by DNA quantification using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). Eight SSR primer pairs (Table 1) were used for polymerase chain reaction (PCR). Information regarding the original source, repeat motifs, primer sequences, expected length, chromosomal localizations and repeat types of the SSRs can be found in the Web database (http://www.gramene.org).

Table 1. Details of the SSR primers used for genetic analysis

Sl. No.	Primer	Sequence	Expected PCR Product Size	Repeat Motif
1	RM1	F-GCGAAAACACAATGCAAAAA R-GCGTTGGTTGGACCTGAC	113	(GA)26
2	RM 261	F-CTACTTCTCCCCTTGTGTCG R-TGTACCATCGCCAAATCTCC	125	C9(CT)8
3	RM 31	F-GATCACGATCCACTGGAGCT R-AAGTCCATTACTCTCCTCCC	140	(GA)15
4	RM 314	F-CTAGCAGGAACTCCTTTCAGG R-AACATTCCACACACACACGC	118	(GT)8(CG)3(GT)5
5	RM 11	F-TCTCCTCTTCCCCCGATC R-ATAGCGGGCGAGGCTTAG	140	(GA)17
6	RM105	F-GTCGTCGACCCATCGGAGCCAC R-TGGTCGAGGTGGGGATCGGGTC	134	(CCT)6
7	RM 244	F-CCGACTGTTCGTCCTTATCA R-CTGCTCTCGGGTGAACGT	163	(CT)4(CG)3C(CT)6
8	RM 229	F-CACTCACACGAACGACTGAC R-CGCAGGTTCTTGTGAAATGT	116	(TC)11(CT)5C3(CT)5

Note: F-Forward sequence, R- Reverse sequence

PCR reaction was carried out in a volume of 10 µl reaction mixture, containing 3 µl of diluted template DNA, 0.5 µl of each forward and reverse primer, 0.25 µl of 10 mM dNTPs, 1.5 µl of 10x buffer, 0.2 µl of *Taq* polymerase, 1.8 µl of $MgCl_2$ and 2.25 µl of ddH_2O and the PCR reaction was carried out in a DNA thermal cycler (G-STORM, GSI, England). The following condition was performed for PCR amplification of SSR marker: 94^0C for 5 min (initial denaturation) followed by 35 cycles of 94^0C for 1 min (denaturation), 55^0C for 1 min (annealing), 72^0C for 2 min (extension) with a final extension for 7 min at 72^0C. After amplification PCR product was mixed with gel loading dye (bromophenol blue, xylene cyanol and sucrose) and electrophoresis was carried out in a mini vertical polyacrylamide gel (8% denatured polyacrylamide gel containing 19:1 acrylamide : bisacrylamide) in TBE buffer. Three micro liters of the sample were loaded in each well and run at 80 volt for 90 minutes. To estimate the PCR product size, 50 bp ladders was used. After completing gel electrophoresis, the gel was stained with ethidium bromide for 30-35 min, kept in dark, and then visualized using gel documentation unit linked to a PC.

Diversity Analysis

Clearly observed unambiguous bands were scored visually for their presence or absence with each primer using Alpha-Ease FC 5.0 software (Alpha Innotech, USA). The number of alleles per locus, major allele frequency, gene diversity and PIC values were calculated using Power Marker version 3.25 (Liu & Muse, 2005). The scores were

obtained in the form of matrix with '1' and '0', which indicate the presence and absence of bands in each variety respectively. This observation was further analysis with NTSYS-pc version 2.2 (Rohif, 2002). NTSYS-pc was used to construct a UPGMA (unweighted pair group method with arithmetic averages) dendrogram showing the distance-based interrelationship among the genotypes.

RESULTS AND DISCUSSION

Morphological characterization

Traditional local rice germplasm, have a high level of genetic diversity compared to modern varieties. This genetic variability is utmost important for the plant breeders, because despite low yield capacity, many germplasm show high tolerant to different abiotic and biotic stresses. Evaluation of morphological traits of these local rice germplasm revealed wide range of variation existed among the genotypes tested, for traits such as number of fertile tiller, 1000 grain weight and yield showing high coefficients of variation (Table 2). The time of flowering is also an important character of rice cultivars and has a direct influence on the success of cross pollination due to synchronization of flowering of parental lines in backcrossing. There were observed low variation (2.2%) for days to 50% flowering where the earliest flowering genotype was Khalia boro (101 days) however Laldinga took as long as 107 days (Table 2). Similar variation in time of flowering was reported by Santhy (1999) where eight rice genotypes were evaluated. The average productive tiller number varied from 6 in Madlai to 10 in Khalia boro with high coefficient of variation of 17.8% (Table 3). Plant height is one of the major traits of rice plant that help for identification of genotypes, assessment of genetic purity as well as the selection of suitable parents. The present study revealed that this character was less variable (1.2%) among the genotypes (Table 2). This study also revealed that most of the germplasms were tall in nature ranging from 107 to 120 cm where the tallest was Tepi boro and Bashful (120 cm) and the shortest was Gofisail (107 cm) (Table 2). Cheema *et al.,* (1987) explained that plant height in rice is a complex character and is the end product of several genetically controlled factors. Rosta (1975) found variation in stem length could be used for identification of off-types in the main field during field inspections. Several workers suggested that time of heading and plant height contribute maximum to the genetic divergence among the genotypes (Singh *et al*., 2006). Panicle length was ranged from 21.43 cm (Chengri boro) to 29.47 cm (Khalia boro) among the rice germplasm tested (Table 2). The results were compared by Sarma *et al*. (2004) where they characterized 142 Aus rice genotypes of Assam and found that eight genotypes showed more than 25 cm panicle length and the remaining genotypes recorded lesser panicle length. Abbasi *et. al*., (1995) suggested that although it contributes positively yet maximum panicle length is not the only factor responsible for higher grain yield. As to maturity, the average growth duration was 137 days ranging from 131-141 days (Table 2). Seed size and shape have been used to differentiate the rice genotypes by various scientists (Agarwal and Karki, 1989; Wang and Shen, 1992); specially thousand grain weights have been used for characterizing rice varieties by several workers and was considered one of the major trait of yield component. Considering 1000-grain weight, Rangila atepi had the maximum thousand grains weight (23.99 g) whereas it was least for Khalia boro (12.57 g) with a higher coefficient variation (17.1%) (Table 2). A variation (7.8%) were found in yield where Thakursail gave the highest yield (4.92 t/ha) with slender grain and lowest in Bashful and Lalia boro (2.08 t/ha) (Table 3) which compared with Hosan *et. al*., (2011) who found grain yield range from 2.01 to 1.41t/ha. The major challenge still exists for plant breeders to develop varieties with shorter duration without sacrificing yield.

Table 2. Mean agronomic performance of eight qualitative traits of 12 rice germplasm

Name of genotypes	DFL (days)	PLH (cm)	PNL (cn)	NFT	GD (days)	FGP	TWG (g)	YLD (t/ha)
Tepiboro	106.00	120.00	25.47	8	141.00	110	23.19	3.59
Rangilaatepi	106.00	111.00	26.47	7	138.00	145	23.99	4.88
Thakursail	106.00	115.33	28.43	7	138.00	158	22.12	4.92
Gofisail	105.00	107.00	25.83	9	139.00	106	23.22	4.24
Laldinga	107.00	118.00	27.30	8	139.00	110	19.44	2.96
Chengriboro	102.00	115.00	21.43	8	131.00	114	19.32	3.29
Jangliboro	102.00	118.00	23.30	8	141.00	94	18.36	2.08
Bashful	104.00	120.67	27.17	9	141.00	145	19.17	4.51
Madlai	104.00	111.67	26.77	6	133.00	122	20.71	2.51
Khaiaboro	104.00	116.00	23.30	9	141.00	83	18.55	2.08
Laliaboro	102.00	116.00	24.80	7	131.00	138	19.29	3.15
Khaliaboro	101.00	118.33	29.47	10	140.00	125	12.57	2.57
$LSD_{0.05}$	3.82	2.27	0.72	2.49	2.65	4.8	5.63	0.21
CV%	2.2	1.2	1.7	17.8	1.1	2.3	17.1	7.8
Probability	ns	**	**	ns	**	**	**	**

Note: DFl- Days to 50% flowering, PLH- Plant height, NFT- Number of productive tiller, GD- Growth duration, FGP- Filled grain per panicle, TWG- 1000 grain weight, YLD- Yrain yield ns-non significant, ** significant at 1 % level of sifignificant

The correlation coefficient among the eight quantitative characters (Table 3) of 12 genotypes revealed that there were positive significant correlation between days to flowering and thousand grain weight (r = 0.700**) and this result is compared with Ullah *et al*. (2011). Grain yield was found to be positively and significantly associated with filled grains per panicle (r = 0.733**) and thousand grain weight (r = 0.622*). The results are supported by Rokonuzzman *et al*. (2008) and Khan *et al*. (2009) for grains per panicle.

Table 3. Phenotypic correlation coefficients among the eigth qualitative traits

	DFL	PLH	NFT	GD	PNL	FGN	TGW	YLD
DFL	1.000							
PLH	-0.164	1.000						
NFT	-0.258	0.191	1.000					
GD	0.338	0.363	0.524	1.000				
PNL	0.310	0.064	0.003	0.266	1.000			
FGN	0.112	-0.012	-0.435	-0.282	0.573	1.000		
TWG	**0.700****	-0.487	-0.558	0.080	-0.127	0.183	1.000	
YLD	0.498	-0.256	-0.215	0.022	0.353	**0.733****	**0.622***	1.000

Note: DFl- Days to 50% flowering, PLH- Plant height, NFT- Number of productive tiller, GD- Growth duration, PNL- Panicle length, FGP- Filled grain per panicle, TWG- 1000 grain weight, YLD- Yrain yield **. Correlation is significant at the 0.01 level (2-tailed), *. Correlation is significant at the 0.05 level (2-tailed). Critical values of Pearson's correlation coefficient at df=35, 0.325 (P = 0.05) and 0.418 (P = 0.01).

Based on agro-morphological traits it can be concluded that most of the germplasm are taller and had moderate filled grain with long panicle, however shortage in productive tiller which ultimately affect grain yield. The genetic variation can reveal the variation of the above discussed quantitative traits and this variability can be used to find raw materials that plant breeder can use to improving present modern variety. However, one must bear in mind that morphological traits are known to be largely influenced by the environment which could result in variation without associated changes at the DNA level (Hillies, 1987).

Molecular characterization

A total of eight SSR markers (Table 1) were used for molecular characterization of 12 Bangladeshi local Boro rice genotypes. Eight microsatellite markers were showed polymorphism (Fig. 1 & Fig. 2) as they were well spread on chromosome 1, 4, 5, 6, 9, 10 and 1(as compared with Sajib et. Al. 2012). The frequencies of an allele at loci are calculated manually by direct counting. The mean number of alleles (MNA) observed over a range of loci for different populations are considered to be a reasonable indicator of genetic variation. A high MNA implies great allelic diversity which could have been influenced by cross breeding. In our study, the number of alleles per locus was ranged from 4 to 7 with an average of 6.125 per locus (Table 5), which was compared with Jain *et al.* (2004) where average of 7.8 alleles per locus ranged from 3 to 22. The number of alleles detected in the study was lower than the average number of alleles reported by Xu *et al.,* (2004), Jayamani *et al.,* (2007), Zeng *et al.,* (2007) and Prathepha *et al.,* (2012) who reported an average of 11.9, 14.6, 7.7 and 11.85 alleles per locus using US rice genetic resources. Furthermore, in a study conducted by Sajib *et al.,* (2012), the number of alleles per locus varied from 2 to 6, with average number of alleles per locus was 3.33 in 12 rice germplasm which was significantly higher than the average alleles found this experiment. Similar lower average were observed by Pervaiz *et al.,* (2010), Upadhyay *et al.,* (2011) and Rahman *et al.,* (2012) who found an average of 4.4, 4.35 and 4.18 alleles per locus.

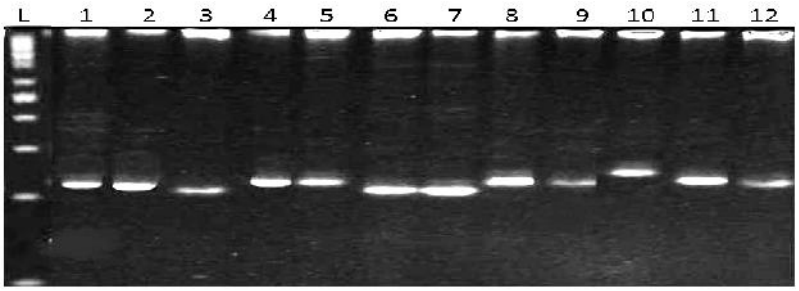

Fig. 1: Banding pattern of 12 rice germplasm using RM 229where Lane L: 50bp ladder; Lane-1: Tepiboro, Lane 2: Rangilaatepi, Lane-3:Thakursail, Lane-4:Gofisail, Lane-5: Laldinga, Lane-6:Chengri boro, Lane-7: Jangliboro, Lane-8: Bash ful, Lane-9: Madlai, Lane-10: Khaiaboro,Lane-11: Laliaboro, Lane-12: Khaliaboro

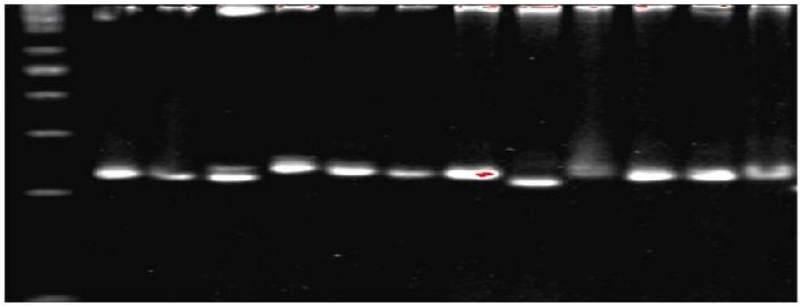

Fig. 2: Banding pattern of 12 rice germplasm using RM 314where Lane L: 50bp ladder; Lane-1: Tepiboro, Lane 2: Rangilaatepi, Lane-3:Thakursail, Lane-4:Gofisail, Lane-5: Laldinga, Lane-6:Chengri boro, Lane-7: Jangliboro, Lane-8: Bash ful, Lane-9: Madlai, Lane-10: Khaiaboro,Lane-11: Laliaboro, Lane-12: Khaliaboro

The PIC values derived from allelic diversity and frequency among the genotypes were not uniform for all the SSR loci tested. The PIC value for 8 primers varied from 0.67 (RM 1) to 0.86 (RM 314) with a mean of 0.76 (Table 4) which was consistent with Borba *et al.*, (2009) and Upadhyay *et al.*, (2011), who were reported average PIC value of 0.75 and 0.78, respectively. Lower PIC value may be the result of closely related genotypes and higher PIC values might be the result of diverse genotypes. In an investigation of genetic diversity in US rice accession, Yunbi *et al.*, (2004) found 714 alleles in the entire data set with average polymorphism information content (PIC) values 0.66 for the SSR markers. Among the primers used in the present study, RM 314 is highly informative (Table 4) since it recorded high PIC value (0.86). The markers showed an average PIC value of 0.76 which indicated that SSR markers used in this study were average informative.

Table 4. Data on the number of major frequency allele, No of allele, genetic diversity and Polymorpism Information Content (PIC) found among the germplasm using 8 microsatellite markers

Marker	Chr. no.	Major Frq. Allele	Number of allele	Genetic Diversity	PIC Value
RM1	1	0.33	4	0.72	0.67
RM 261	4	0.42	5	0.74	0.70
RM 31	5	0.25	7	0.83	0.81
RM 314	6	0.17	9	0.88	0.86
RM 11	7	0.33	5	0.75	0.71
RM105	9	0.33	6	0.78	0.75
RM 244	10	0.17	7	0.85	0.83
RM 229	11	0.42	6	0.75	0.72
Mean		0.30	6.1250	0.79	0.76

Genetic distance- based analysis

The genetic similarity analysis was done using UPGMA clustering. Cluster analysis showed significant genetic variation among the germplasm tested. The UPGMA clustering system generated four genetic clusters with a genetic distance of 0.28 (Fig. 3). Here Rangila atepi and Thakur sail were clustered in same group but closer to Chengri boro and Gofisail, Laldinga and Tepi boro (Fig. 3). Four germplasm viz. Khalia boro, Madlai, Khaia boro and Bashful were clustered distinctly in same group. Again Janngli boro and Lalia boro were in same cluster (Fig. 3). These results indicate that although these germplasm showed morphological differences, they could have originated from much related sources with similar DNA sequences. Thus morphology based analysis alone is not sufficient due to environmental effects on phenotypic characters.

In summary, morphological and molecular characterization both could serve as a potential basis in the identification of genetically distant accessions as well as in identification of the morphologically close accessions. This characterization also provided valuable information about genetic diversity of 12 local Boro rice germplasm. Therefore, it is highly necessary not only to conserve germplasm, but also to reveal the gene-pool of rice germplasm and unlock valuable genes for breeding purposes. However, the use of more number of markers would be efficient to characterize the germplasm than used for the study, which highlighted the presence of diversity at genomic level among the genotypes studied.

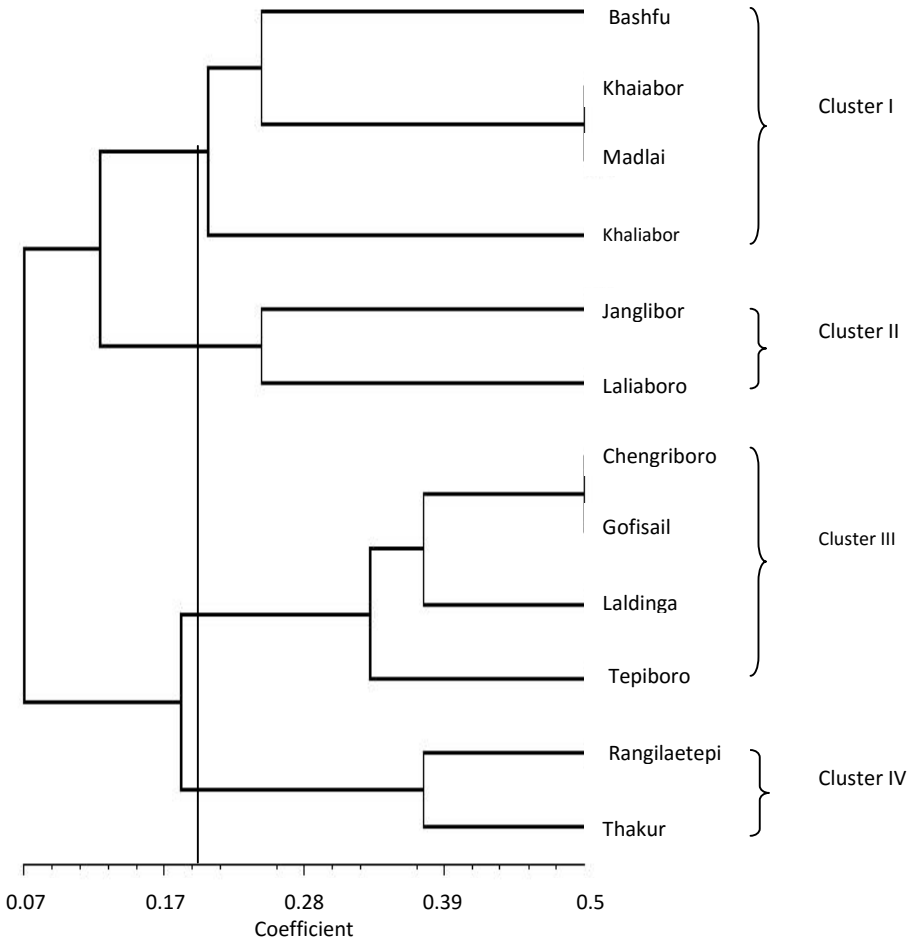

Fig. 3. A UPGMA clustering dendogram showing the genetic relationships among twelve local boro rice germplasm based on the alleles detected by 8 microsatellite markers.

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