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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOME ADVANCED MUTANT RICE (*Oryza sativa* L.) GENOTYPES

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the main cereal crops in the world and the main staple food of Bangladesh. Amylose content of rice endosperm is the main chemical properties that contributes the eating and cooking quality. On the other hand, rice protein is the important source of nutrition of almost 50 per cent of the world's population living on rice. A total of 12 rice genotypes including ten advanced mutant lines, one landrace namely Laxmidigha along with BRRI dhan49 was collected from Plant Breeding Division of Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh and Bangladesh Rice Research Institute (BRRI), Gazipur. The genotypes were analyzed to assess the amylose and protein content as well as their genetic diversity relationship. Among all the tested genotypes amylose content were ranged from 23.9-20.3% where highest amylose percentage was found for Magic-86 (25.6%). Protein content of the genotypes were ranged from 8.50-5.04%, where highest 8.86% protein was recorded for SSB-3. The highest gene diversity (0.81) was observed in RM519 marker and the lowest (0.28) was in RM111 marker. The PIC values ranged from 0.24-0.78 with a mean of 0.51. The highest Nei's genetic distance value 0.87 was found in RM-16(N)-10 vs RM-40(C)-4-2-8 and the lowest value 0.18 was found in LD-200-1-3-3-8 vs LD-200-1-3-2-4. A dendogram was constructed using UPGMA system based on Nei's similarity coefficient and six major clusters were obtained at 0.7 cut off. The genotypes showing diverse ranges of amylose and protein content tended to cluster together in the dendogram with some exceptions.

Keywords: Amylose, Genetic diversity, Protein, Rice, SSR marker

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INTRODUCTION

Rice is a major cereal crop grown as a staple food exclusively for over half of the world's population. It is a semi-aquatic grass plant belongs to the genus *Oryza* in the Gramineae (Poaceae) family. South Asia has been described as the "food basket" and "food bowl" of Asia because of being one of the major centers for rice domestication. Overall milled rice production in South Asia has reached 16.9 crore tons (USDA, 2021). Bangladesh has become the third rice growing countries with the production of 3.6 crore tons after China and India that produce 14.6 crore tons and 11.8 crore tons, respectively (USDA, 2021). Being the major crop, rice engages more than 70% of the rural population and the national economic system of Bangladesh (Anonymous, 2002). But the population is growing approximately 1.20% per year (BBS, 2018). If this rate of increasing population continues, the total population will be approximately 238 million by 2050 (Shelly et al., 2016). Hence rice production needs to increase to meet the demand of food for the growing population.

Rice grain quality is an important attribute for the rice scientists, producers, and consumers as stated by Riza et al. (2018). The quality of rice is significantly affected by the two attributes i.e., amylose and protein contents (Champagne et al., 1998). The amylose content is related to the stickiness or of the cooked rice. Low amylose containing rice becomes tender and glossy after cooking while high amylose restricts rice to absorb water making the cooked rice dry, fluffy and detached (Juliano, 1971). The protein content determines the texture of the cooked rice by hindering absorption of the water while cooking (Xie et al., 2008). Rice breeders are consistently anxious regarding having new rice lines with suitable amylose and protein contents. Protein is an essential component required for growth, antibodies production and immunity in human beings. Rice is a vital source of protein, delivering additional 50% of the entire protein consumed in the most of the countries. Market value of many varieties depends on the quality of the rice in many countries (Fitzgerald et al., 2008; Champagne et al., 1999).

Generally, rice genotypes are recognized and identified based on morpho-biochemical traits. Molecular characterization along with morphological traits would be the best solution. By characterization at the molecular level, similarity or distance from parent or check material containing particular traits can be more precisely defined. Molecular markers can reveal abundant difference among genotypes at the DNA level. They provide a more direct, reliable, and efficient tool for germplasm characterization, screening and evaluation. In rice, micro-satellites are abundant and well distributed throughout the genome (Wuand Tanksley, 1993; Akag et al., 1996; McCouch et al., 1997). They are valuable as genetic markers because they are co-dominant in nature, detect high levels of allelic diversity and are assayed efficiently by the PCR technique (McCouch et al., 2002). Therefore, the current study aims to estimate protein and amylose content of the mutant lines along with their characterization of the genetic diversity using SSR markers.

MATERIALS AND METHODS

Materials

A total of 12 rice genotypes (ten mutant genotypes, one landrace and one check variety BRRI dhan49) were studied in the experiment. RM-16 (N)-10, RM- Kas-80(C)-1, LD-200-1-3-3-5, LD-200-1-3-3-8, LD-200-1-3-2-4, RM-40(C)-4-2-8, SSB-3, Magic-10, Magic-72, Magic-86 and Laxmidigha were collected from Bangladesh Institute of Nuclear Agriculture (BINA) and BRRI dhan49, which was collected from Bangladesh Rice Research Institute (BRRI).

Estimation of amylose and protein content

At first, 100 g seeds of each milled rice genotype were ground well by a grinder. After that the rice powder of each genotype was sieved separately by 120 mesh sieves. Finally, the powder of each genotype was taken in separate zipper bags and stored in a desiccator for amylose and protein content estimation. Amylose content was estimated by iodine colorimetry at a wavelength of 620 nm and using a potato starch standard (Juliano et al., 1981; Ambardekar et al., 2011). Protein content was estimated by Kjeldahl method (AOAC, 1995).

Preparation of standard amylose curve

At first 1N acetic acid, iodine solution was poured in 5 separate 100 ml volumetric flasks as well as a blank for calibration (Table 1). Then the six volumetric flasks were filled up to 100 ml. The mixtures were measured at 620 nm in a spectrophotometer and a standard amylose curve was prepared by using the absorbance reading with different amylose equivalence. Each sample solution was measured at 620 nm to get absorbance reading. Finally, amylose content of each sample was determined by calculating with the standard amylose curve.

Table 1. Reaction mixtures for standard amylose curve

Sl. No.	1N Acetic acid (ml)	Iodine sol^{n} (ml)	Standard stock sol^{n} (ml)	Amylose Equivalence (%)
1	-	2	-	0
2	0.4	2	1	8
3	0.8	2	2	16
4	1.2	2	3	24
5	1.6	2	4	32
6	2.0	2	5	40

Analysis of protein content by conventional Kjeldahl method

Total N% and Protein% in each sample were found from the reading of UKD 159 Automatic Distillation and Titration System. N:P conversion factor 5.95 for rice was used in this system (Greenfield and Southgate, 1992).

Molecular characterization

Selection of SSR markers

Total 16 SSR markers (Table 2) were used for genetic diversity analysis in this study.

Table 2. Genetic details of 16 SSR markers

Markers Name	Product Size(bp)	Chromosome No.	Sequences	Annealing temp. (°C)
RM 217	133	6	Forward: ATCGCAGCAATGCCTCGT Reverse: GGGTGTGAACAAAGACAC	55
RM 42	166	8	Forward: ATCCTACCGCTGACCATGAG Reverse: TTTGGTCTACGTGGCGTACA	55
RM 237	130	1	Forward: CAAATCCCGACTGCTGTCC Reverse: TGGGAAGAGAGCACTACAGC	55
RM 431	251	1	Forward: TCCTGCGAACTGAAGAGTTG Reverse: AGAGCAAAACCCTGGTTCAC	55
RM 307	174	4	Forward: GTACTACCGACCTACCGTTCAC Reverse: CTGCTATGCATGAACTGCTC	55
RM 105	134	9	Forward: GTCGTCGACCCATCGGAGCCAC Reverse: TGGTCGAGGTGGGGATCGGGTC	55
RM 171	328	10	Forward: AACGCGAGGACACGTACTTAC Reverse: ACGAGATACGTACGCCTTTG	55
RM 228	154	10	Forward: CTGGCCATTAGTCCTTGG Reverse: GCTTGCGGCTCTGCTTAC	55
RM 206	147	11	Forward: CCCATGCGTTTAACTATTCT Reverse: CGTTCCATCGATCCGTATGG	55
RM 536	243	11	Forward: TCTCTCCTCTTGTTTGGCTC Reverse: ACACACCAACACGACCACAC	55
RM 519	122	12	Forward: AGAGAGCCCCTAAATTTCCG Reverse: AGGTACGCTCACCTGTGGAC	55
RM 286	110	12	Forward: GGCTTCATCTTTGGCGAC Reverse: CCGGATTCACGAGATAAACTC	55
RM 111	124	6	Forward: CACAACCTTTGAGCACCGGGTC Reverse: ACGCCTGCAGCTTGATCACCGG	55
RM 475	235	2	Forward: CCTCACGATTTTCCTCCAAC Reverse: ACGGTGGGATTAGACTGTGC	55
RM 168	116	3	Forward: TGCTGCTTGCCTGCTTCCTTT Reverse: GAAACGAATCAATCCACGGC	55
RM 11	140	7	Forward: TCTCCTCTTCCCCCGATC Reverse: ATAGCGGGCGAGGCTTAG	55

Genomic DNA extraction

Healthy portion of the young, vigorous leaves from 21 days old seedling was cut apart with sterilized scissors and washed in 70% ethanol and distilled water. The samples were stored at -20°C freezer. Cetyl Trimethyl Ammonium Bromide (CTAB) method was used to extract DNA from the leaves of each genotype (Doyle and Doyle 1987). PCR amplification, confirmation and documentation of genomic DNA were proceeded for the diversity analysis of the genotypes (Hoque et al., 2021).

DNA amplification by Polymerase Chain Reaction (PCR)

The PCR cocktail was placed in the PCR tubes and operated in the DNA thermal cycler, with a total volume of 10 µl/reaction mixture containing 1 µl of DNA based on the IRRI rice protocol of SSR analysis. A 1.5 ml PCR tube was filled with 3 µl of sterilized ddH2O and 5 µl of Go Taq Green master mix. Then 0.5 µl of forward primer and 0.5 µl of reverse primer were added together. After vortexed, 1 µl of diluted DNA sample was pipetted into the PCR tube wells and the tube was centrifuged for 30-40 seconds to ensure proper mixing. Thus, a total of 10 µl of PCR sample was prepared. The system was then run according to the instruction of Thermal cycler by the following PCR profile: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, primer elongation at 72°C for 1 min and then a final extension at 72°C for 5 min. Amplified products were incubated at 4°C until electrophoresis.

Confirmation of DNA using gel electrophoresis

The genomic DNA was confirmed by polyacrylamide gel electrophoresis. A flask was loaded with 50 ml premix, 650 µl 10% APS and 72 µl TEMED (N,N,N',N'-tetramethylethane-1,2-diamine) and mixed well by magnetic stirrer. The gel was then carefully poured into the gel mold and combs were laid out on the gel. After solidifying in the gel tank, the gel was immersed in 0.5X TBE buffer. The gel was then ready for loading the DNA samples. Then using a micropipette, loading dye (4 µl) and extracted DNA sample (2 µl) were thoroughly mixed and placed into the gel's slot. The first lane of the gel was loaded with a recognized DNA marker (DNA ladder). The electrophoresis apparatus was connected to the power supply unit and the gel tank was submerged in 1X TBE. Electrophoresis was conducted for 60 minutes at 70 volts.

Documentation of the DNA samples

After electrophoresis, the gel was carefully removed from the gel chamber and stained with a previously prepared ethidium bromide solution (0.25%). After 30 minutes, the gel was carefully removed from the staining tray and placed on the gel doc's high-performance ultraviolet light box (UV trans-illuminator) to check the DNA bands. Using Alpha Ease 4.0 software, the DNA was identified as a band and the records were saved.

Data analysis

The experiments were conducted through CRD design. The recorded data for amylose and protein content were analyzed using MSTAT-C package program (Gomez and Gomez, 1984). Power marker version 3.23 (Liu and Muse, 2005), a genetic analysis software was used to calculate statistical results such as the number of alleles per locus, major allele frequency, gene diversity, and polymorphism information Content (PIC) values. The Alpha Ease 4.0 software was used to calculate the molecular weights of microsatellite products in base pairs. The alleles of the relevant microsatellite loci were assigned to the individual fragments. The genetic distance or phylogeny reconstruction based on neighbor-joining method (Saitou and Nei, 1983) as implemented in the software with the tree viewed using tree view was also determined using allele molecular weight data.

RESULTS AND DISCUSSION

Estimation of amylose and protein content

The amylose content of selected 12 rice genotypes/variety was determined with reference to the standard amylose curve **(**Fig. 1). Standard amylose curve was obtained from amylose equivalence and the absorbance of the standard mixtures at 620 nm in a spectrophotometer.

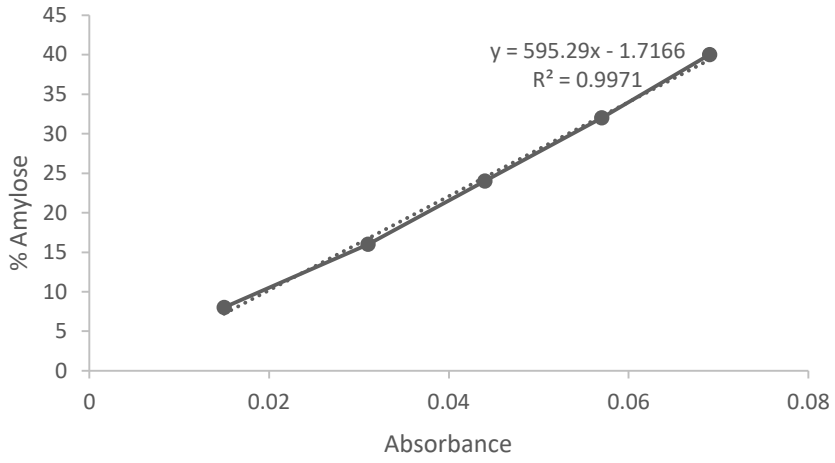

Figure 1. Standard amylose curve

The amylose content of the genotypes varied from 20.3-25.6%. The highest amylose content (25.6%) was found in Magic-86; followed by RM-40(C)-4-2-8 (25.1%) and Magic-10 (24.4%). There were 10 groups (Table 3) in which the means were significantly different from one another at 5% level of significance. Critical Value for Comparison was 0.168. These values were similar as reported by Paul (2020) where amylose percentage for rice was analyzed.

Significant variation in the protein content of the genotypes were observed. The highest protein content (8.86%) was found in SSB-3; followed by Magic-86 (8.78%) and Laxmidigha (8.73%). There were nine groups (Table 3) in which the means were significantly different from one another at 5% level of significance. These protein contents were similar to those reported by Magomya et al. (2014)

Table 3. Amylose and protein contents of 12 rice genotypes

Sl. No.	Name of the genotypes	Amylose (%)	Protein %
1	RM-16 (N)-10	20.3j	6.12g
2	RM- Kas-80(C)-1	22.1g	5.79h
3	LD-200-1-3-3-5	21.5h	8.32e
4	LD-200-1-3-3-8	23.3e	8.41d
5	LD-200-1-3-2-4	22.1g	8.28e
6	RM-40(C)-4-2-8	25.1b	5.04i
7	SSB-3	22.7f	8.86a
8	Magic-10	24.4c	7.47f
9	Magic-72	23.9d	8.50c
10	Magic-86	25.6a	8.78ab
11	BRRI dhan49	23.9d	7.49f
12	Laxmidigha	20.9i	8.73b
	CV (%)	0.44	0.59
	LSD (0.05)	0.168	0.07

Molecular characterization of the rice genotypes using SSR markers

The analysis of genetic variation is a very important factor for the development of rice lines, which can be achieved through DNA profiling techniques that displays high amount of loci for large variability. The sample of rice cultivars collected from various origins were analyzed using SSR markers which is basically a PCR based technique. In this SSR marker-based DNA fingerprinting technique, the rice genotypes were analyzed using 16 loci. Amplified microsatellite loci were analyzed to find out diversity. All 16 microsatellite loci had 6alleles (mean). The bands obtained were compared to the band of BRRI dhan49 and Laxmidigha (Fig. 2, Fig. 3).

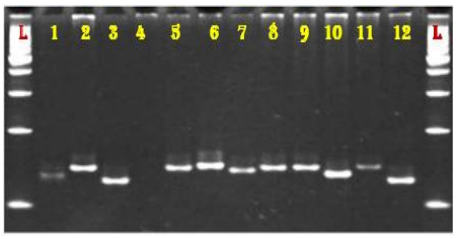

Figure 2. Microsatellite profiles of 12 rice genotypes at locus RM519

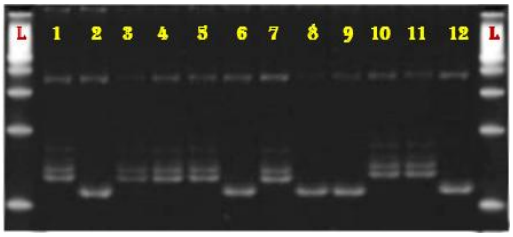

Figure 3. Microsatellite profiles of 12 rice genotypes at locus RM11

Legend:1= RM-16 (N)-10, **2**= RM- Kas-80(C)-1, **3**= LD-200-1-3-3-5, **4**= LD-200-1-3-3-8, **5**= LD-200-1-3-2-4, **6**= RM-40(C)-4-2-8, **7**= SSB-3, **8**= Magic-10, **9**= Magic-72, **10**= Magic-86, **11**= BRRI dhan49, **12**= Laxmidigha, **L**=25 bp ladder.

Gene diversity

The allele with the highest frequency (more than 10 percent) is termed as major allele or most common allele at each locus. The size of various major alleles at different loci ranges from 115bp (RM286) to 322bp (RM171). Among all the genotypes, on an average, 54% of them shared a common major allele ranging from 25% (RM105 and RM519) to 83% (RM307 and RM111) at each locus (Table 4). The result was more or less similar to the result reported by Siddique et al. (2016). The highest gene diversity (0.81) was observed in RM519 and the lowest (0.28) was in RM111, having an average diversity of 0.55. It was found that marker detecting the higher number of alleles showed higher gene diversity, on the other hand lower number of alleles expressed lower gene diversity (Table 4). Heenan et al. (2000) also found similar result of gene diversity.

The PIC value reflects allele diversity and frequency among the genotypes. PIC value of each marker can be determined based on its allele. PIC varied significantly for all the studied SSR loci. In this study, the level of polymorphism among rice genotypes was evaluated by calculating PIC values for each of the 16 SSR loci. The PIC values ranged from 0.24 to 0.78, having an average of 0.51 per locus. According to Jiang et al. (2010), a PIC value of greater than 0.5 is reflective of a good marker. The highest PIC value was 0.78 for RM519 and the lowest was 0.24 for RM111 in this study (Table 4).

Table 4. Major allele, gene diversity and PIC value found among selected rice genotypes for 16 SSR markers

Marker	Major Allele		Allele No.	Gene Diversity	PIC
	Size (bp)	Frequency (%)			
RM 42	163	41	5	0.65	0.58
RM 237	130	67	8	0.51	0.48
RM 431	238	67	8	0.49	0.42
RM 307	170	83	10	0.29	0.27
RM 105	137	25	3	0.78	0.74
RM 171	322	33	4	0.72	0.67
RM 228	142	58	7	0.58	0.53
RM 206	152	33	4	0.74	0.69
RM 536	246	41	5	0.65	0.58
RM 519	125	25	3	0.81	0.78
RM 286	115	75	9	0.40	0.36
RM 111	124	83	10	0.28	0.24
RM 475	235	75	9	0.40	0.36
RM 168	118	58	7	0.58	0.53
RM 11	143	58	7	0.49	0.37
Mean		54	6	0.55	0.51

Nei's (1973) genetic distance

The pair wise comparison values of Nei's (1973) genetic distance among 12 rice genotypes were calculated from combined data sets for 16 loci. The value ranged from 0.18 to 0.87 (Table 5). The highest Nei's genetic distance value 0.87 was found in RM-16(N)-10 vs RM-40(C)-4-2-8. The lowest genetic distance value 0.18 was found in LD-200-1-3-3-8 vs LD-200-1-3-2-4. The lowest value of pair wise difference among rice genotypes was likely due to their genetic relatedness. On the other hand, higher value of pair-wise difference was observed among those rice lines developed from genetically distal parental. Similar result was found by Lakhanpaul et al. (2000) where a total of 267 amplification products were formed at an average of 12.71 per primer with an overall polymorphism of 64%. The extent of polymorphism was moderate to low.

Table 5. Summary of genetic distance values among selected rice genotypes using 16 SSR markers

Gen.*	P1	P10	P11	P12	P2	P3	P4	P5	P6	P7	P8	P9
P1	0.00											
P10	0.75	0.00										
P11	0.56	0.56	0.00									
P12	0.81	0.62	0.56	0.00								
P2	0.81	0.81	0.68	0.56	0.00							
P3	0.50	0.68	0.31	0.50	0.50	0.00						
P4	0.75	0.62	0.43	0.50	0.43	0.43	0.00					
P5	0.62	0.68	0.43	0.56	0.43	0.31	0.18	0.00				
P6	0.87	0.75	0.56	0.62	0.37	0.68	0.37	0.43	0.00			
P7	0.75	0.68	0.50	0.62	0.68	0.62	0.43	0.43	0.62	0.00		
P8	0.75	0.62	0.62	0.56	0.62	0.68	0.50	0.62	0.50	0.50	0.00	
P9	0.68	0.50	0.43	0.62	0.62	0.62	0.50	0.62	0.50	0.43	0.37	0.00

Here, P1= RM-16 (N)-10, P2= RM- Kas-80(C)-1, P3= LD-200-1-3-3-5, P4= LD-200-1-3-3-8, P5= LD-200-1-3-2-4, P6= RM-40(C)-4-2-8, P7= SSB-3, P8= Magic-10, P9= Magic-72, P10= Magic-86, P11= BRRI dhan49, P12= Laxmidigha.

Gen.*= Genotypes used in this study.

Genetic similarity analysis using UPGMA

Dendogram based on Nei's (1973) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated differentiation of the 12 rice genotypes by 16 markers. All the 12 rice genotypes could be easily distinguished. The UPGMA cluster analysis led to the grouping of 12 rice genotypes in six major clusters at 70% cut off (Fig. 4).

Cluster-1, Cluster-2 and Cluster-4 each considered only one genotype and they were RM-16 (N)-10, Magic-86 and Laxmidigha respectively, which differed from other genotypes in amylose and protein content. Cluster-3 consists of 3 genotypes having two sub clusters (3A and 3B). In 3A, SSB-3 and in 3B, Magic-10 and Magic-72 were clustered. These genotypes found to contain moderate amylose and protein content. In cluster-5, 2 genotypes namely RM- Kas-80(C)-1 and RM-40(C)-4-2-8 clustered together, and they all were grouped in relatively low protein rice. Cluster-6 considered two sub clusters (6A and 6B). Sub cluster 6A comprised of 2 genotypes, LD-200-1-3-3-8 and LD-200-1-3-2-4, those were found as moderate amylose and high protein content. Sub cluster 6B contains BRRI dhan49 and LD-200-1-3-3-5 those were identified as moderate amylose containing rice. The genotypes showing diverse ranges of amylose and protein content tend to cluster together in the

dendogram with some exceptions. Siddique et al. (2016) also observed six clusters among 20 rice genotypes of Bangladesh using 30 SSR markers.

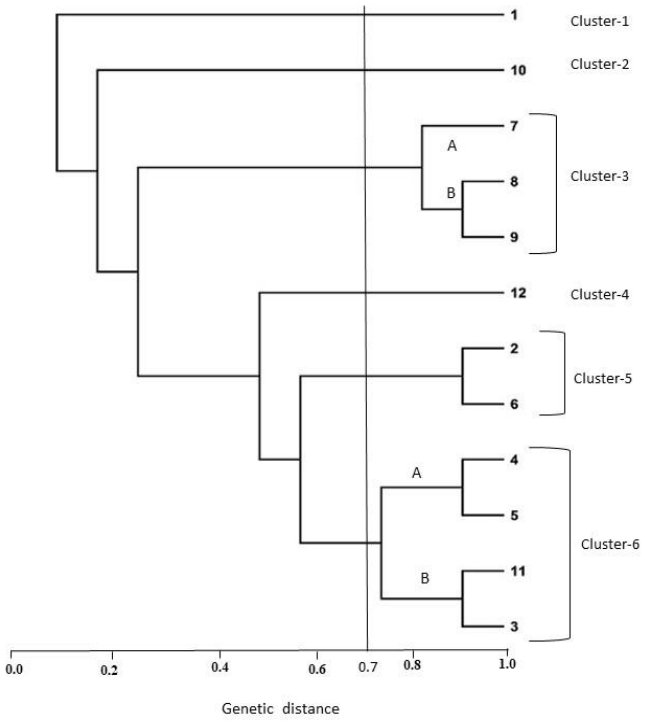

Figure 4. UPGMA Dendogram based on Nei's Genetic Distance according to SSR analysis

Legend:1= RM-16 (N)-10, 2= RM- Kas-80(C)-1, 3= LD-200-1-3-3-5, 4= LD-200-1-3-3-8, 5= LD-200-1-3-2-4, 6= RM-40(C)-4-2-8, 7= SSB-3, 8= Magic-10, 9= Magic-72, 10= Magic-86, 11= BRRI dhan49, 12= Laxmidigha.

CONCLUSION

Among 12 rice genotypes, the amylose content varied from 20.3- 25.6%. The highest amylose content (25.6%) was found in Magic-86 and the highest protein content (8.86%) was found in SSB-3. In this study, genetic diversity, and relationship among 12 rice genotypes at molecular level was addressed using 16 SSR markers. Genetic distance among 12 rice genotypes were calculated from combined data sets for 16 loci. Genetically similar genotypes with the checks can be utilized developing trait specific characters for further breeding programs. Based on the results it can be concluded that, Magic-86, SSB-3 and LD-200-1-3-3-8 can be used to develop better quality rice varieties. Field trial of the suggested genotypes may help breeders to develop potential rice varieties in future.

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