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Assessment of Genetic Diversity in Rice Germplasm (*Oryza sativa* L.) using SSR Markers

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ABSTRACT

Thirty eight rice accessions were analyzed to evaluate the genetic polymorphism and identification of diverse parents using simple sequences repeat (SSR) markers. These accessions showed significant phenotypic variation for all the characters studied. A total of 61 alleles were detected by 11 polymorphic markers showing highly polymorphic across all accessions with an average of 5.54 alleles per polymorphic marker. Five of the markers RM324, RM171, RM31, RM24, RM30 produced maximum 6 alleles. The PIC value ranged from 0.275 to 0.751 with a mean PIC of 0.633 and Markers RM31, RM336, RM30 and RM280 were the most informative primers to discriminate among the accessions on the basis of highest PIC of 0.751, 0.735, 0.731 and 0.719, respectively. The cluster analysis showed that these accessions grouped in to seven clusters in which cluster IIB had maximum 8 genotypes followed by cluster IA-1 (7 germplasms) and cluster IA-2 (6 germplasms). While, highest dissimilarity coefficient value was observed between the cultivar cultivar C-3 and LAL KHADHA. These accessions were showing wide genetic divergence among the constituent in it and may be directly utilized in hybridization programme for improvement of yield related traits.

Introduction

Rice is the life and the prince among cereals as this unique grain helps to sustain two thirds of the world's population (Bisen *et al.,* 2019). The rice accessions are a rich reservoir of useful genes that rice breeder can harness for rice improvement programme and the genetic variability exists among rice accessions leaving a wide scope for crop improvements (Rashmi *et al.,* 2017; Singh *et al.,* 2015). Genetic diversity is a pre-requisite for any

crop improvement breeding programme as it helps in estimating and establishing the genetic relationship in the collection of accessions, identifying diverse parental combinations to create segregating progenies with maximum genetic variability and superior recombinations (Thompson *et al.,* 1998; Islam *et al.,* 2012; Ramadan *et al.,* 2015). Parents identified on the basis of divergence would be more promising for breeding program (Kwon *et al.,* 2002). Genetic diversity is mainly measured based on the morphological differences of quantitative important traits.

However, this method has some disadvantages in terms of time, space, and labour cost. In addition, this method cannot define the exact level of genetic diversity among the germplasms, because of the additive gene action on the expression of the traits (economically important traits), thus making environmental factors mask their true phenotypic performance (Aljumaili *et al.,* 2018; Schulman 2007; Zeng *et al.,* 2004).

Use of molecular markers is the chromosomal landmark through which an organism can be recognized and has gained popularity as a genetic diversity tool (Ishii *et al.,* 2001). Evaluation of genetic diversity using DNA marker technology is non-destructive, not affected by environmental factors, requires small number of samples, and does not require large experimental setup (Rashmi *et al.,* 2017; Kanawapee *et al.,* 2011).

Among the PCR-based markers, the SSR markers have proved to be very effective tools in the study of genetic diversity and organism relationships due to their high polymorphic nature and transferability (He *et al.,* 2003). SSR markers even in less number can give a better genetic diversity spectrum due to their multi-allelic and highly polymorphic nature (Singh *et al.,* 2016). Due to replacement of native varieties with high yielding varieties as a result of green revolution, extreme losses in diversity has been occurred in rice germplasms (Choudhary *et al.,* 2013; Heal *et al.,* 2004).

Therefore, the present study was undertaken with the aim to assess the analyze the genetic diversity in rice accessions using SSR markers. The generated information will enable maximized selection of diverse parents and selecting appropriate parental genotypes in breeding programme.

Materials and Methods

The experiment was carried out during *kharif* season 2016 at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. The experimental seed material comprises of thirty eight rice accessions provided by DBT Networking Project (Table 1).

The nursery was sown on 14th June, 2016 on uniform raised beds applied with a fertilizer dose of 1.0 Kg N, 1.0 Kg P_2O_5 and 0.5 Kg K_2O per 50 m^2 area. 21 days old seedling was transplanted in a randomized block design (RBD) with three replications by maintaining row to row and plant to plant spacing 20 × 15 cm, respectively.

Genomic DNA extraction

Young leaves of 15-20 days old seedlings from sixty five rice genotypes were clipped and stored in ice-box to carry it to the lab which is then stored in -800C till DNA extraction. Genomic DNA was then extracted using CTAB method (Doyle and Doyle 1987). DNA samples were diluted to 10 ng/µl. The DNA was quantified spectrophotometrically (PerkinElmer, Singapore) by measuring A260/A280 and DNA quality was checked by electrophoresis in 0.8% agarose gel.

SSR markers and PCR amplification

Twelve (12) SSR markers were used for genotyping the entire 38 rice accessions (Table 2). Total PCR reaction was optimized to be 15 µl and this included 1 µl of about 50 ng DNA template, 7.0 µl DreamTaq PCR master mix (Thermos Scientific Inc.), 1 µl of each primer (forward and reverse primer), and 5.0 µl nuclease free water. Thermal cycling program involved an initial denaturation at 94° for 45 sec, annealing at 2° bellow Tm of respective primers for 30 sec, primer extension at 72° for 30 sec, followed by a final extension at 72° for 7 min. The band separation was done by running the PCR products on 2.5% agarose at 80 v for 60 min in 1% TBE along with 50 bp DNA ladder. The gel was viewed using Bio-Rad gel documentation machine. The gel picture was analyzed using Bio-Rad Image lab software for the band size. The data were saved in Excel for further analysis.

SSR data analysis

The effects of different scales of measurement for different quantitative traits were minimized by standardizing the data for each trait separately prior to cluster analysis. Standardization was done by dividing the deviation of mean for a line from the mean for thirty eight lines with the standard deviation for the given trait; the STAND module of NTSYS (Rohlf, 1997) software was used to furnish the same. The binary data matrix generated by polymorphic SSR markers were subjected to further analysis using NTSYS-pc version 2.11W (Rohlf, 1997). The SIMQUAL programe was used to calculate the Jackard' dissimilarity coefficient. The dissimilarity matrix was used as an input for analysis of clusters. UPGMA-based clustering was done using SAHN module of NTSYSpc for dendogram construction. In Unweighted pair-group average (UPGMA) clusters are joined based on the average distance between all members in the two groups. PIC for SSR markers was calculated as per the formula:
$PIC_i = 1-\sum Pij2$,

Where, PIC_i = polymorphic information content of a marker i.
P_{ij}= frequency of the jth pattern for marker i.
And, $\sum$ extends over n patterns.

PCA was also done to check the result of UPGMA base clustering using EIGEN moduleof NTSYSpc. The first step in PCA is to calculate eigen values, which define the amount of total variation that is displayed on Principal Component axis. The first PC summarizes most of the variability not summarized by, and uncorrelated with, the first PC, and so on. PCs were used for 2-dimentional (2-D) and 3-dimentional (3-D) plotting, respectively, against each other using module PROJ and MXPLOT of NTSYSpc.

Results and Discussion

Polymorphism and marker efficiency

Thirty eight rice genotypes were subjected to SSR marker assay to assess the molecular diversity. Out of twelve markers used, eleven showed polymorphism and were reproducible suitable for diversity analysis while, one primer (RM224) was not amplified. The eleven polymorphic primers yielded a total of 61 fragments (amplified products). The size of fragments varied from 76bp (RM30) to 350bp (RM171). Maximum fragments were produced by primer RM336 which showed 7 fragments. Gel images showing SSR banding profile obtained by primer RM324, is presented in figure 1. The polymorphic information content (PIC) was employed for each locus to assess the information of each marker and its discriminatory ability. The level of polymorphism among rice genotypes was evaluated by calculating allelic number

and PIC values for each of the eleven polymorphic SSR markers (Table 3). A total of 61 alleles were detected by 11 polymorphic markers across thirty eight rice accessions with an average of 5.54 alleles per polymorphic marker. Among the polymorphic markers, 5 produced 6 alleles each, 4 markers have produced 5 alleles each and 2 of them produced 4 and 7 alleles each. The number of alleles observed in this study was higher than findings of (Gowda *et al.,* 2012; Singh *et al.,* 2000). On the other hand, higher number of alleles as much as 6.60 to 14.60 have been reported using other rice varieties (Thomson *et al.,* 2007). A total of 128 alleles with an average of 3.28 alleles per locus and PIC value of 0.24 were observed by Kibria *et al.,* (2008) using 39 SSR markers. The number of alleles indicates the richness of the population (Aljumaili *et al.,* 2018). Since SSR are short tandem repeats, generally allele numbers of 2 to 7 alleles per locus are considered good as seen in this study. The amplicon size varied from 76bp produced by RM30 to 350bp produced by marker RM171. PIC value is a reflection of allele diversity and frequency among genotypes. The PIC value observed in the present investigation ranged from 0.27 to 0.75 with a mean PIC of 0.63; comparable to previous estimates of microsatellite analysis in rice viz., 0.21 to 0.79 with a mean of 0.46 (Ramadan *et al.,* 2015) and 0.25 to 0.98 with an average of 0.61 (Aljumaili *et al.,* 2018). Markers RM31, RM336, RM30 and RM280 were the most informative primers on the basis of highest PIC of 0.751, 0.735, 0.731 and 0.719 respectively. SSR marker RM324 showed least PIC value of 0.275. The higher the PIC value of a locus, the higher the number of alleles detected. High PIC as seen in this study revealed that the markers have the required properties to be used in diversity study. RM31 was found to be the most appropriate marker to discriminate among the rice genotypes owing to the highest PIC value of 0.751.

Table.1 List of thirty eight rice genotype used in present study

S.No.	Genotype	S.No.	Genotype
1	C-1	20	**R1570-418-1-149-1**
2	C-2	21	**NDR-1159**
3	C-3	22	**UPRI 2012-15**
4	C-6	23	**RPBIO4918-70-11**
5	C-7	24	**IR 83142-60**
6	SANGSANGBA	25	**HURLC 22**
7	MOIRANG PHOU	26	**IR82635-B-B47-1**
8	MOIRANG PHOU KHONG NEMBT	27	**BVS 1**
9	KUMBI PHOU	28	**IC 256649**
10	LANG PHOU	29	**CR 3635-1-1**
11	LOKTAK PAT PHOU	30	**JALNIDHA**
12	TUMAT AMUBI	31	**LAL KHADHAN**
13	BARASALI	32	**IC 426013**
14	IR78933-B24-B-B-4	33	**HURL-54**
15	HURLC-40	34	**DHAULAKISH**
16	CR-3488-1-2-1-2	35	**CSR-36**
17	CRR599-51	36	**IC 337598**
18	OR2172-7-1	37	**TETEP(Check)**
19	**CRR599-4-1**	**38**	**IC 337598**

Table.2 Details of the SSR primers used in present study

S.No.	SSR Primer	Sequence	Chr. No.	Tm(ºC)
1.	RM24 F	GAAGTGTGATCACTGTAACC	1	**58.3**
	RM24 R	TACAGTGGACGGCGAAGTCG		
2.	RM324 F	CTGATTCCACACACTTGTGC	2	**53.3**
	RM324 R	GATTCCACGTCAGGATCTTC		
3.	RM231 F	GGTGATCCTTTCCCATTTCA	3	**55.6**
	RM231 R	CACTTGCATAGTTCTGCATTG		
4.	RM280 F	ACACGATCCACTTTGCGC	4	**57.4**
	RM280 R	TGTGTCTTGAGCAGCCAGG		
5.	RM31 F	GATCACGATCCACTGGAGCT	5	**58.35**
	RM31R	AAGTCCATTACTCTCCTCCC		
6.	RM30 F	GGTTAGGCATCGTCACGG	6	**59.8**
	RM30 R	TCACCTCACCACACGACACG		
7.	RM336 F	CTTACAGAGAAACGGCATCG	7	**57.3**
	RM336 R	GCTGGTTTGTTTCAGGTTCG		
8.	RM152 F	GAAACCACCACACCTCACCG	8	**59.65**
	RM152 R	CCGTAGACCTTCTTGAAGTAG		
9.	RM242 F	GGCCAACGTGTGTATGTCTC	9	**58.55**
	RM242 R	TATATGCCAAGACGGATGGG		
10.	RM171 F	AACGCGAGGACACGTACTTAC	10	**58.5**
	RM171 R	ACGAGATACGTACGCCTTTG		
11.	RM224 F	ATCGATCGATCTTCACGAGG	11	**56.4**
	RM224 R	TGCTATAAAAGGCATTCGGG		
12.	RM17 F	TGCCCTGTTATTTTCTTCTCTC	12	**58.55**
	RM17R	**GGTGATCCTTTCCCATTTCA**		

Table.3 Allele size (bp) and polymorphism information content (PIC) of the SSR primers used in the present study

S.No.	Primers	No. of Alleles amplified	Approx. size of amplified product(bp)	PIC
1	RM32	6	150-172	**0.275**
2	RM231	5	160-192	**0.421**
3	RM280	5	150-182	**0.719**
4	RM336	7	132-200	**0.735**
5	RM242	4	200-236	**0.641**
6	RM152	5	140-160	**0.714**
7	RM171	6	300-350	**0.672**
8	RM31	6	132-160	**0.751**
9	RM24	6	150-300	**0.656**
10	RM17	5	100-140	**0.655**
11	**RM30**	**6**	**76-112**	**0.731**

Table.4 Grouping of thirty eight rice germplasm into different clusters based on Jaccard's IJ coefficient

Cluster	Number of Genotypes	Name of the Genotypes
IA	5	**C-1, C-3, C-6, C-7, MOIRANG PHOU**
IB-1a	4	**C-2, LANG PHOU, TUMAI AMUBI, BARASALI**
IB-1b	3	**MOIRANG PHOU KHONG NEMBT, KUMBI PHOU, SANGSANGBA**
IB-2	5	**LOKTAK PAT PHOU, CR-3488-1-2-1-2, IR73933-B24-B-B-4, HURLC-40, OR 2172-7-1**
IIA-1	7	**CRR 599-51, TETEP(Check), CRR 599-4-1, HURLC-54, IC 256649, DHAULAKISH, IR82635-B-B47-1**
IIA-2	6	**UPRI 2012-15, HURLC 22, RPBIO 4918-70-11, CR 3635-1-1, IC 337598, JALNIDHA**
IIB	**8**	**R 1570-418-1-149-1, IC 337598, BVS1, IC 426013, CSR-36, NDR-1159, IR 83142-60, LAL KHADHAN**

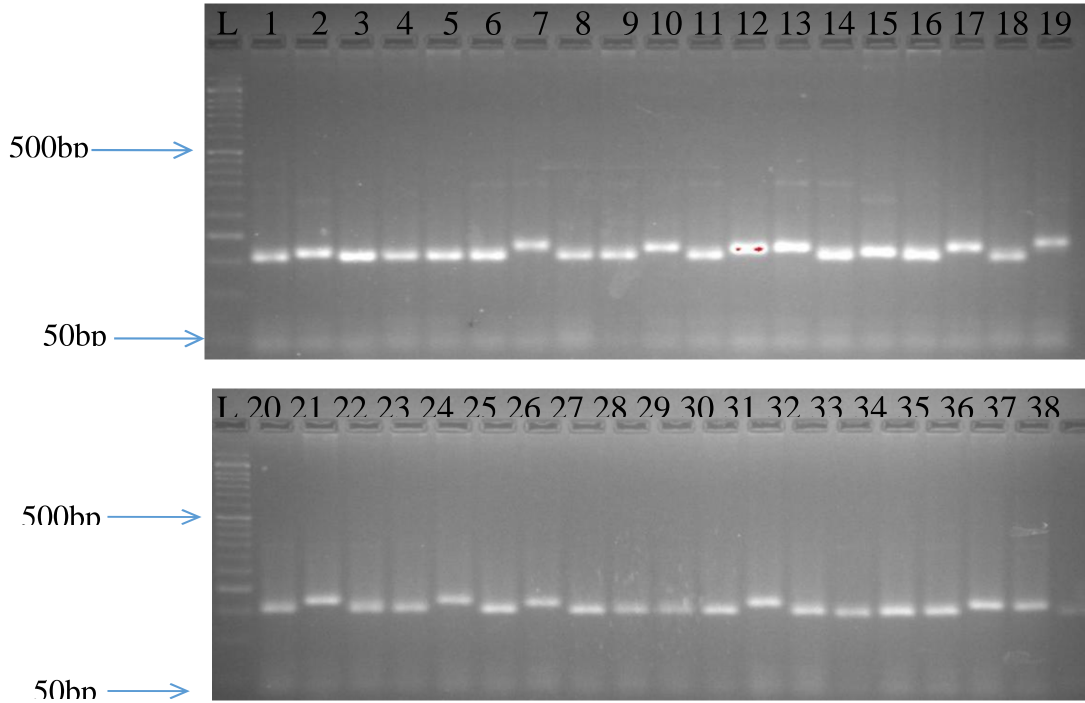

Fig.1 SSR banding profile obtained by marker RM324. Lane 1-38 represents rice cultivar used in the present study; M=50bp DNA size marker

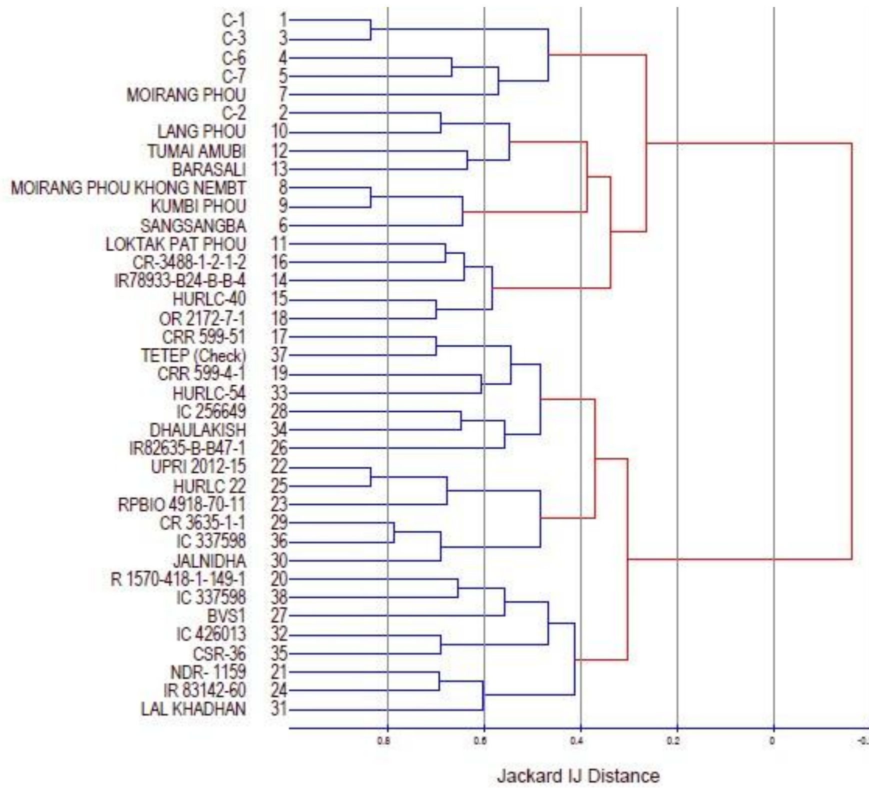

Fig.2 Cluster dendogram of thirty eight genotype on the basis of molecular markers

Dendogram analysis

According to this dissimilarity coefficient we can understand the dendogram and their relatedness. So, highest diverse genotypes can be used as parents in breeding programme. A dendogram based on Jackard's dissimilarity coefficient was constructed using UPGMA (Fig. 2). Thirty eight rice germplasms were grouped into two main clusters i.e. cluster I and cluster II (Table 4). Cluster I was further sub-divided into two minor sub-groups IA and IB with dissimilarity coefficient (0.27). Cluster IB were further sub-divided into two minor subgroups i.e. IB-1 and IB-2 (0.30). Cluster IB-1 was also sub-divided into two minor subgroups i.e. IB-1a and IB-1b with dissimilarity coefficient (0.39). Cluster II was also sub divided in to two minor sub-groups i.e IIA and IB with dissimilarity coefficient (0.35) and cluster IIA was further sub-divided in to two subgroups i.e IIA- 1 and IIA-2 (0.38). This indicated presence of considerable diversity in the germplasms studied. The most diverse genotype is therefore, important in order to select desirable genotypes for utilizing in breeding programmes.

The dissimilarity coefficient varies from 0 to 1, close to one shows high dissimilarity while close to 0 shows high similarity. The average of dissimilarity coefficient varies from 0.78 to 0.92. The total average of dissimilarity coefficient of all thirty eight cultivars is 0.85. The dissimilarity coefficient varied from the largest value 1.00 between the cultivar C-3 and LAL KHADHA which shows high dissimilarity between them. The lowest value 0.33 was found between C-1 and C-3 followed by 0.33 between the cultivar MOIRANG PHOU KHONG NEMBT and KUMBI PHOU showing that they are highly similar from each other. The most diverse cultivar was C-3 and LAL KHADHA. Similar result was found by Siva *et al.,* (2010).

Genetic diversity is an important concept in any breeding program. It can be studied using SSR markers for the identification of potential parent in order to achieve heterosis in future rice breeding program. SSR markers were exploited to provide an true estimate of the diversity pattern in the rice germplasm.

A total of 61 alleles were detected by 11 polymorphic markers across 38 rice genotypes with an average of 5.54 alleles per polymorphic marker. The amplicon size varied from 76bp produced by RM30 to 350bp produced by marker RM171. PIC value ranged from 0.27 to 0.75 and marker RM31 was found to be the most appropriate marker to discriminate among the rice genotypes owing to the highest PIC value of 0.75.The genetic divergence study grouped 38 rice genotypes into seven clusters in which cluster IIB had maximum eight genotypes followed by cluster IIA-1 and cluster IIA-2.

On the basis of dendogram the highest similarity observed between cultivar C-1 and C-3 followed by MOIRANG PHOU KHONG NEMBT and KUMBI PHOU.. According to dissimilarity coefficient, the highest dissimilarity coefficient value was observed between the cultivar C-3 and LAL KHADHA (1.00) and between whereas lowest value was seen between C-1 and C-3(0.33) showing highly similar genotypes. Thus, these accessions were genetically diverse and could be directly utilized in hybridization programme for improvement of yield related traits or to execute efficient selection in highly segregating generations.

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