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Molecular marker based genetic diversity analysis in rice (*Oryza sativa* L.) using SSR markers

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Abstract

Assessment of genetic diversity is very important in rice breeding from the standpoint of selection, conservation and proper utilization. The role of a broad genetic base and systematically characterized germplasm in the crop improvement of cultivated plants has been well recognized. To meet the continuously expanding needs of varietal improvements, the evaluation and characterization of all existing germplasm are required. Genetic variability studies are important in selection of parents for hybridization as sound crop improvement depends upon the magnitude of variability in the base population. In the present investigation 36 rice genotypes were studied for diversity studies using polymorphic SSR markers. These rice genotypes were grouped into two main clusters that is cluster I and II with similarity coefficient (0.00). Cluster I can be sub divided into two minor sub-groups IA, IB, and with similarity coefficient (0.15). Cluster IA consisted of 12 genotypes, whereas cluster IB consisted of 13 genotypes. The second main cluster can be also sub divided into two minor sub-groups that is IIA and IIB (0.25). This indicated presence of considerable diversity in the genotypes studied. Through diversity analysis, highest similarity was observed between cultivar URG-5 and URG-8 followed by NDR-359 and Pusa-6-B. The most diverse cultivars were Akshaya dhan and URG-24.

Highlights

- The highest similarity observed between cultivar URG-5 and URG-8.
- The most diverse cultivar was Akshayadhan and URG-24.
- This indicated presence of considerable diversity in the genotypes studied

Keywords: Genetic diversity, conservation, germplasm, similarity coefficient and dendrogram

Rice (*Oryza sativa* L.) belongs to the genus *Oryza* of family Poaceae. The genus *Oryza* is known to consist of two cultivated species i.e. Asian rice (*Oryza sativa*, 2n=24=AA) and African rice (*Oryza glaberrima*, 2n=24=AA) and 22 wild species (2n=24, 48). Asian rice has undergone differentiation into three distinct eco-geographical subspecies, *viz., indica, japonica* and *javanica* (Singh *et al*. 2000; Vaughan *et al*. 2003). South East Asia is supposed to be the original home of *Oryza sativa* (Vaughan 1994). From its place of origin, rice spread to various parts of the world and is presently food for a major part of human population. About half of the world population depends on rice for their survival. It is cultivated in 114 a country across the globe, but 90 percent of world's rice is grown in Asia. India has the largest area under rice among the rice growing countries in the world and ranks second in production after China.

Rice is grown in almost all the states of India, but major rice producing states fall in the regions of middle and lower gangetic plains, as well as

the coastal lowlands of peninsular India. Being a leading producer of rice, there is a need of more production per unit area to fulfill the needs of ever growing population. In order to meet this, hybridization is a prime consideration for which the diversified genotypic assessment has to be done.

Assessment of genetic diversity is important in plant breeding for its improvement. For the assessment of genetic diversity molecular markers have been generally superior to morphological, pedigree, heterosis and biochemical data (Melchinger *et al*. 1991). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Weir, 1990). Molecular Marker based Genetic Diversity Analysis (MMGDA) also has potential for assessing changes in genetic diversity over time and space (Duwick, 1984).

A molecular marker is a DNA sequence that is readily detected and whose inheritance can be easily monitored. Recently, DNA profiles based on various molecular markers have been widely applied across different fields. Molecular markers are powerful tools for evaluation of genetic diversity. Of the wide array of DNA markers available, microsatellite or simple sequence repeat (SSR) markers are considered to be appropriate for assessment of genetic diversity and variety identification because of their ability to detect large numbers of discrete alleles repeatedly, accurately and efficiently. Microsatellite markers have been ideal for identification and purity checking of rice varieties. Microsatellites are PCR-based markers, they are abundant, co-dominant, highly reproducible and interspersed throughout the genome (Panaud *et al*. 1996, Temnykh *et al*. 2000). In particular, they are able to detect high levels of allelic diversity. These markers can detect a significantly higher degree of polymorphism in rice which becomes ideal for studies on genetic diversity and intensive genetic mapping. Simple sequence repeat is an important tool for genetic variation identification of germplasm (Powell *et al*. 1996). Considering the potential applications of MMGDA, a study was made involving a set of 36 genotypes of rice to assess the level of genetic diversity among the rice cultivars released over a period of time and other genotypes.

Materials and methods

The present study was carried out during. *kharif*-2014 (at the Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, U.P., India). The molecular analysis was accomplished at the Molecular Biology Laboratory (Niche Area Lab) of the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (U.P.), India. The experimental material for this investigation comprised of 36 genotypes. A total of four simple sequence repeat (SSR) markers, *i.e.*, RM315, RM443, RM171 and RM6100 were used for studying molecular diversity. The details of SSR primers used are presented in Table 1.

Table 1: Details of the microsatellite primers used in present study

Microsatellite locus	Location/ Chromosome	Forward/Reverse	Sequence 5′----------> 3′
RM315	1	Forward	GAGGTACTTCCTCCGTTTCAC
		Reverse	AGTCAGCTCACTGTGCAGTG
RM443	1	Forward	GATGGTTTTCATCGGCTACG
		Reverse	AGTCCCAGAATGTCGTTTCG
RM171	10	Forward	AACGCGAGGACACGTACTTAC
		Reverse	ACGAGATACGTACGCCTTTG
RM6100	10	Forward	TCCTCTACCAGTACCGCACC
		Reverse	GCTGGATCACAGATCATTGC

Isolation of DNA

Young leaves were collected from 12 days old rice seedlings and immediately stored at -20 ^{0}C till further processing. The DNA was extracted following CTAB extraction method according to Doyle and Doyle (1987) with few modifications in composition of DNA extraction buffer.

DNA quality estimation

The DNA quality estimation was done using Biophotometer plus. The ratio (OD_{260}/OD_{280} ratio) thus obtained was used to estimate the nucleic acid purity in the different DNA samples. A ratio of 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids. A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers. A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) samples were re-precipitated to purify the DNA.

Polymerase chain reaction (PCR)

Polymerase chain reaction was performed to selectively amplify in vitro a specific segment of the total genomic DNA to a billion fold (Mullis *et al*. 1986). The most essential requirement of PCR is an availability of a pair of short (typically 20-25 nucleotides) primers having sequence complementary to either end of the target DNA segment (called template DNA) supposed be synthesized in large amount. The PCR conditions standardized for the present experiment are presented in the Table 2.

Table 2: Standardized concentration of the PCR components

PCR component	Stock concentration	Final concentration	Volume for 15 µl
Primer (F + R)	10 pM	0.7 pM	2.0 (1.0 + 1.0)
Taq DNA Polymerase	5U/µl	1U	0.4
$Mgcl_2$	25 mM	0.3 mM	0.2
Taq Assay Buffer	10x	1x	1.5
dNTPs	10 mM	0.14 mM	0.2
Genomic DNA	50 -100 ng/µl	50 ng /15µl reaction vol.	2.0
HPLC water	-	-	8.7

For adjustment of concentration of various chemicals, amount of $MgCl_2$ was changed keeping other PCR components as constant. For PCR programming all the steps were kept as such except the annealing temperature. Annealing temperature was determined based on the GC content of the primer as:

$$Tm = [2 \times (A+T) + 4 \times (G+C)] - 4$$

This formula provided the preliminary information but not the exact annealing temperature. Therefore, the correct annealing temperature was determined based on best PCR amplification. All the amplifications were performed in the Eppendorf Thermo-cycler (USA) using the thermal cycler program. After the completion of the PCR, the products were stored at -20ºC until the gel electrophoresis was done.

Scoring the PCR amplified fragments

Band position in comparative SSR profile for each genotype and primer combination was scored from the respective gel images. SSR profile from only that genotype × primer combination, which gave constant amplification for all the genotype and without any blank lane per unclear bands, was included in this study. The amplified fragments were scored as '1' for the presence and '0' for the absence of a band generating the 0 and 1 matrix. These binary data matrix was then utilized to generate genetic similarity data among the 36 lines of rice genotypes.

Statistical analysis

Standardization of quantitative data

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 36 genotype with the standard deviation for the given trait; the STAND module of NTSYS software was used to furnish the same.

Genetic Similarity

The binary data matrixes generated by polymorphic SSR markers were subjected to further analysis using

NTSYS-pc version 2.11W. The SIMQUAL programe was used to calculate the jackard's coefficient.

Cluster analysis based on UPGMA

The similarity matrix was used as an input for analysis of clusters. UPGMA-based clustering was done using SAHN module of NTSYSpc for dendrogram construction. In Unweighted pair-group average (UPGMA) clusters are joined based on the average distance between all members in the two groups.

Polymorphic information content (PIC)

PIC for SSR markers was calculated as per the formula: PIC= 1-Pij2, where PICi is the polymorphic information content of a marker i and the summation extends over n patterns. PCA was also done to check the result of UPGMA base clustering using EIGEN module of NTSYSpc.

Results and discussion

Molecular diversity in rice is done by using SSR markers. It is the complimentary sequences of DNA which lie close to the particular gene or QTL. So by annealing the primer we can amplify our target region close to the gene. In present investigation the study of diversity among thirty six rice cultivars was done by using four polymorphic rice SSR markers. All markers showed polymorphism and cluster analysis was done to construct dendogram using Jaccard's similarity coefficient.

Diversity analysis

A dendograme (Figure 1) based on Jaccard's similarity coefficient were constructed using UPGMA based cluster analysis. Thirty six rice genotypes were grouped into two main clusters that is cluster I and II with similarity coefficient (0.00). Cluster I can be sub divided into two minor sub-groups IA, IB with similarity coefficient (0.15). Cluster IA consisted of 12 genotypes, whereas cluster IB consisted of13 genotypes. The second main cluster can be also sub divided into two minor sub-groups that is IIA and IIB (0.25). This indicated presence of considerable diversity in the genotypes studied. The most diverse genotype is therefore, important in order to select desirable genotypes for utilizing in breeding programmes. On the basis of dendogram the highest similarity observed between cultivar URG-5 and URG-8 followed by NDR-359 and Pusa-6-B. The most diverse cultivar was Akshaya dhan and URG-24.

Similarity coefficient

The similarity coefficient varied from genotypes Akshaya dhan and URG-24. The genotype URG-20 and URG-21 are more similar in origin due to their low similarity coefficient. The genotypeNDR-359 and Pusa-6-B are more similar in origin due to their low similarly coefficient, genotype Akshaya dhan is distantly related to URG-19 with the similarity coefficient of (0.25) and genotype Vandana is distantly related to URG-3 with the similarity coefficient 0f (0.40), genotype Pant dhan-12 distantly related to HUR-3022 with the similarity coefficient of (0.50) and genotype Vandana showed least similarity with BPT-5204 and highest similarity with Anjali followed by Baranideep. The genotype Akshaya dhan is least similar to IR-64 with similarity coefficient of (0.62) and showed highest similarity with URG-42 followed by URG-28. Similarly genotype Sahabhagi showed least similarity with Vardhan with (0.25) similarity coefficient and should highest similarity with Nagina-22 and followed by HUR-10-9. Similar results were found by Singh *et al.* (1999) for Boro rice, genetic divergence in Boro rice (Sinha *et al*. 1991), genetic divergence in indigenous upland rice varieties, Souroush *et al*. (2004). Percent contribution of 4 rice markers towards divergence is presented in table 4. All the primers used were polymorphic (Plate 1-4) among which RM 171 (0.94), RM 6100 (0.90) and RM 443 (0.93), RM (0.85) markers showed higher diversity comparatively.

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

Primer	PIC Value
Primer 171	0.94
Primer 6100	0.90
Primer 315	0.85
Primer 443	0.93

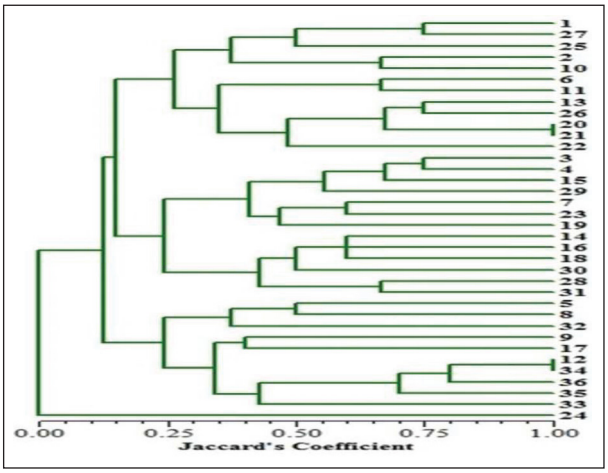

Fig. 1: Dendrogram depicting the relationship of 36 genotypes of rice

1=AkshayaDhan, 2= Danteswari, 3= Vandana, 4= Anjali,5= Sahabhagi ,6= B.G.-102, 7=NDR – 97 , 8=Nagina – 22, 9=ShuskSamrat , 10= IR-64 , 11= IR-36 , 12= NDR- 359 , 13= MTU – 1010, 14=Pant Dhan -12 , 15= Baranideep, 16= Lalat , 17= Birsa Dhan-105 , 18=URG -1 , 19=URG -3, 20=URG -5 , 21=URG -8 , 22=URG -19 , 23=URG -22 , 24=URG -24 , 25=URG -28 , 26=URG -30 , 27=URG -42 , 28=IET – 22202 , 29=BPT – 5204 , 30=HUR – 105 , 31=HUR – 3022 , 32=HUR -10-9 , 33=Vardhan , 34=Pusa-6-B , 35=IR-68897-B , 36=IR-79156 –B

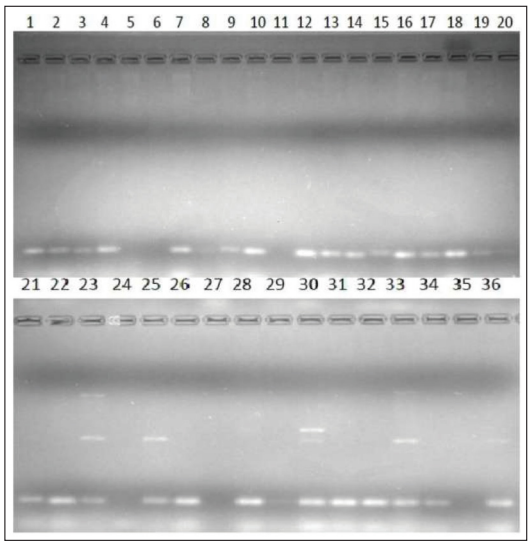

Plate 1: Showing SSR banding profile obtained by marker RM 315. Lane 1-36 represents rice cultivar

1=AkshayaDhan, 2= Danteswari, 3= Vandana, 4= Anjali, 5= Sahabhagi, 6= B.G.-102, 7=NDR – 97, 8=Nagina – 22, 9=ShuskSamrat, 10= IR-64, 11= IR-36, 12= NDR- 359, 13= MTU – 1010, 14=Pant Dhan -12, 15= Baranideep, 16= Lalat, 17= Birsa Dhan-105, 18=URG -1, 19=URG -3, 20=URG -5, 21=URG -8, 22=URG -19, 23=URG -22, 24=URG -24, 25=URG -28, 26=URG -30, 27=URG -42, 28=IET – 22202, 29=BPT – 5204, 30=HUR – 105, 31=HUR – 3022, 32=HUR -10-9, 33=Vardhan, 34=Pusa-6-B, 35=IR-68897-B, 36=IR-79156 –B

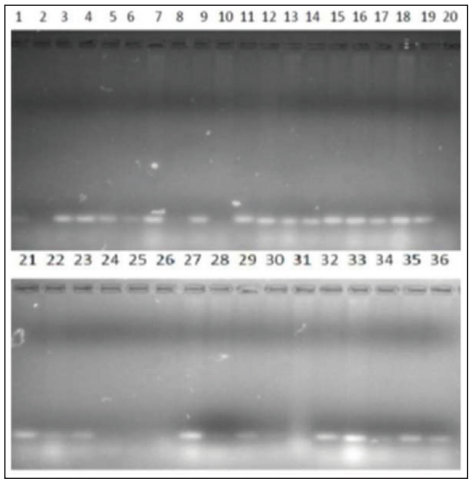

Plate 2: Showing SSR banding profile obtained by marker RM 443. Lane 1-36 represents rice cultivar

1=AkshayaDhan, 2= Danteswari, 3= Vandana, 4= Anjali, 5= Sahabhagi, 6= B.G.-102, 7=NDR – 97, 8=Nagina – 22, 9=ShuskSamrat, 10= IR-64, 11= IR-36 , 12= NDR- 359 , 13= MTU – 1010, 14=Pant Dhan -12, 15= Baranideep, 16= Lalat, 17= Birsa Dhan-105, 18=URG -1, 19=URG -3, 20=URG -5, 21=URG -8, 22=URG -19, 23=URG -22, 24=URG -24, 25=URG -28, 26=URG -30, 27=URG -42, 28=IET – 22202, 29=BPT – 5204, 30=HUR – 105, 31=HUR – 3022, 32=HUR -10-9, 33=Vardhan, 34=Pusa-6-B, 35=IR-68897-B, 36=IR-79156 –B

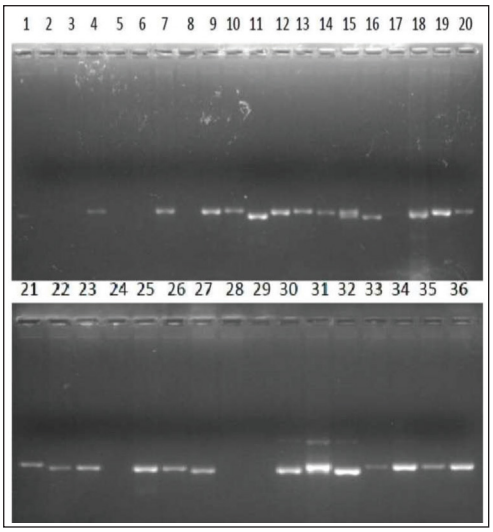

Plate 3: Showing SSR banding profile obtained by marker RM 171. Lane 1-36 represents rice cultivar

1=AkshayaDhan, 2= Danteswari, 3= Vandana, 4= Anjali, 5= Sahabhagi,6= B.G.-102, 7=NDR – 97, 8=Nagina – 22, 9=ShuskSamrat, 10= IR-64, 11= IR-36, 12= NDR- 359, 13= MTU – 1010, 14=Pant Dhan -12, 15= Baranideep, 16= Lalat, 17= Birsa Dhan-105, 18=URG -1, 19=URG -3, 20=URG -5, 21=URG -8, 22=URG -19, 23=URG -22, 24=URG -24, 25=URG -28, 26=URG -30, 27=URG -42, 28=IET – 22202, 29=BPT – 5204, 30=HUR – 105, 31=HUR – 3022, 32=HUR -10-9, 33=Vardhan, 34=Pusa-6-B, 35=IR-68897-B, 36=IR-79156 –B

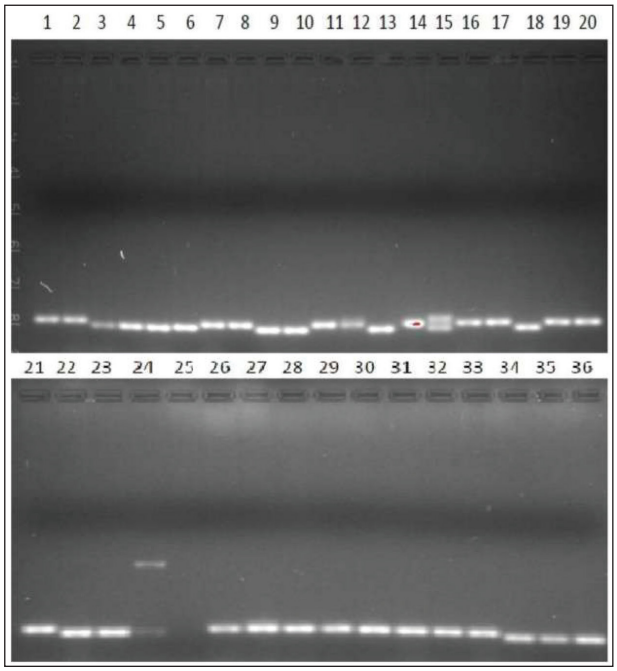

Plate 4: Showing SSR banding profile obtained by marker RM 6100. Lane 1-36 represents rice cultivar

1=AkshayaDhan, 2= Danteswari, 3= Vandana, 4= Anjali,5= Sahabhagi, 6= B.G.-102, 7=NDR – 97, 8=Nagina – 22, 9=ShuskSamrat, 10= IR-64, 11= IR-36, 12= NDR- 359, 13= MTU – 1010, 14=Pant Dhan -12 , 15= Baranideep, 16= Lalat, 17= Birsa Dhan-105, 18=URG -1, 19=URG -3, 20=URG -5 , 21=URG -8 , 22=URG -19 , 23=URG -22 , 24=URG -24, 25=URG -28, 26=URG -30, 27=URG -42, 28=IET – 22202, 29=BPT – 5204, 30=HUR – 105, 31=HUR – 3022, 32=HUR -10-9, 33=Vardhan, 34=Pusa-6-B, 35=IR-68897-B, 36=IR-79156 –B

PIC value

The polymorphic information content (PIC) was employed for each locus to assess the information of each marker and its discriminatory ability. The calculation of PIC (Weir, 1996) for the *i*th marker is PIC = 1 – Σ*Pij* (*j*= 1,2,..., n), where *Pij* is the frequency of the *j*th pattern for the ***i***th marker and the summation extends over (n) patterns (Peng and Lapitan, 2005).The PIC value is an evidence of diversity and frequency among the varieties (Pervais *et al*., 2009). The PIC value of each marker can also be evaluated on the basis of its alleles and diverse for all SSR loci. The largest PIC value was observed for locus RM 171(0.94) followed by RM 443 (0.93) and lowest by RM 315 (0.85) followed by RM 6100 (0.90). So PIC value ranged from 0.94 to 0.85 with a mean value of 0.92. All the 4 primers showed polymorphism and the number of alleles ranged from 2 to 4 with an average of 3.62. A total of 124 microsatellite alleles were amplified from 36 cultivars and this demonstrates considerable variability among cultivar. The PIC values observed in this study were comparable to those reported in some studies (M. Ashfaq and A. S. Khan (2013); Saini *et al*. 2004) but higher than those reported by Singh *et al*. (2005).

Conclusion

Thirty six rice genotypes were grouped into two main clusters that is cluster I and II with similarity coefficient (0.00). Cluster I can be sub divided into two minor sub-groups IA, IB, and with similarity coefficient (0.15Cluster IA consisted of 12 genotypes, whereas cluster IB consisted of 13 genotypes. The second main cluster can be also sub divided into two minor sub-groups that is IIA and IIB (0.25). This indicated presence of considerable diversity in the genotypes studied.

On the basis of dendogram the highest similarity observed between cultivar URG-5 and URG-8 followed by NDR-359 and Pusa-6-B. The most diverse cultivar was Akshaya dhan and URG-24.

The largest PIC value was observed for locus RM 171(0.94) followed by RM 443 (0.93) and lowest by RM 315 (0.85) followed by RM 6100 (0.90).

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