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Original scientific article

GENETIC DIVERSITY ANALYSIS OF SELECTED LOCAL RICE VARIETIES OF PAKISTAN USING RAPD MARKERS

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Assessment of the genetic diversity is very important for the improvement of many crop species including rice. The study was undertaken to assess the genetic diversity prevailing among ten commercially grown rice varieties of Pakistan using nine random amplified polymorphic DNA (RAPD) markers. All the nine primers were able to produce a total of 444 amplicons with an average of 49. 33 amplicon per primer. A total of 356 polymorphic amplicons were generated and showed 80.18 % polymorphism with an average number of 39.55 polymorphic bands per primer. RAPD marker, GL C-12 produced maximum number of bands (76 in all varieties) whereas GL G-14 generated minimum number of bands (26) in the genomic pool. A high level of genetic polymorphism at the DNA level was observed among the ten *Oryza sativa* varieties with an average genetic distance ranging from 28% to 64% on the basis of average dissimilarity coefficient matrix following UPGMA. A dendrogram constructed using the information of the average dissimilarity coefficient matrix of all the ten varieties based on the data of nine RAPD primers placed the varieties in two categories. Cluster analysis grouped six rice varieties i.e. Sarshar, TN-1, Jajai-77, JP-5, Pakhal and Shadab into one group, indicating the similarities between these varieties while the four rice varieties RI-DR-92, Shaheen Basmari, NIR-9 and Sada Hayat were found to be different from the six varieties grouped together. The cluster analysis placed RI-DR-92 and Sarshar in the different groups

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confirms the maximum genetic distance between the two varieties as shown by the genetic distance (64%) estimated in percent. The dendrogram indicated that the RI-DR-92 and Sarshar and JP-5 and Sada Hayat are distantly apart from one another and can be crossed to broaden the genetic base of *Oryza sativa*. In addition, more informative primers can be converted to sequence tagged sites (STS) and sequence characterized amplified regions (SCAR) for the amplification of specific alleles which can aid further in rice genome analysis.

Keywords: RAPD marker, Genetic diversity, Polymorphism, Rice, Cultivars

INTRODUCTION

Rice rank one of the chief food crops in the world, as it is used to nourish almost half of the human population mostly in developing countries (WANG *et al.*, 2018). For millions of people worldwide, it is a staple food source, and it is essential in combating hunger and food insecurity (CGRI, 2017). This is important to mention that approximately 92% of the total global rice is produced by countries such as China, Indonesia, Pakistan, India, Vietnam, Thailand, Myanmar, Bangladesh, Philippines, and Japan (SIDDIQUE *et al*., 2024). In terms of both production and exports, Pakistan makes a substantial contribution to the global rice market. It ranks 10th globally in terms of rice production and fourth globally in terms of exports, behind Thailand, Vietnam, and India (FAO, 2018).

Apart from its economic impact, rice has become a vital plant for genetic and genomic studies. Additionally, because of its diploid and small genome size, genetic polymorphism, vast collection of genetically diverse and conserved genetic material of approximately 100,000 accessions, and availability of compatible wild species, rice is considered an example for studying grass genetics (NAAZ *et al*., 2022). However, it is worth mentioning that the genotypes of rice vary greatly both within and between landraces (SINGH *et al*., 2018). For crop improvement, breeder scan chooses desirable qualities from the diversity of rice accessions to generate novel combinations (GARRIS *et al*., 2005).

Numerous methods facilitate the assessment of genetic diversity at both the genotypic and phenotypic levels. At the genotypic level, molecular markers are one of the best ways to analyze variety in rice since they can detect significant variations between accessions at the DNA level, making them a more dependable and well-thought-out tool for genetic make-up and accession characterization. There are numerous similar techniques, including ISSR, AFLP, SSR, RAPD, and others (NAAZ *et al*., 2022). However, molecular marker-based RAPD method has many discrete benefits over isozyme and other DNA fingerprinting technologies due to its fast data-acquisition, low prices of chemical reactions, small quantity of plant material needed and the capability to perform analyses without the need for preceding sequencing of the genome (VIEIRA *et al*., 2022; BABU *et al*., 2021). With having these advantages, RAPD analysis has become a famous and effective technique for analysis of genetic polymorphism in different rice cultivars. MAZUMDER *et al*. (2020) earlier found 100% polymorphism among Bangladeshi native cultivars by using RAP primer. This study has shown that rice varieties can be successfully distinguished using RAPD primers based on DNA banding characteristics.

Estimation of genetic variability is vital for the better plant selection in plant breeding. RAPD markers technique is being used by various researchers to estimate the genetic diversity in

numerous cultivated rice varieties, aromatic rice and local landraces rice (REKHA *et al*., 2011; HASAN and RAIHAN, 2015; MAZUMDER *et al*., 2020). The aim of this study was to estimate the genetic variability among 10 local rice varieties of Pakistan employing RAPD markers. This data will help the plant breeders to readily select the diverse group of rice cultivars which will add new germplasm base for rice breeding programs in future.

MATERIAL AND METHODS

Plant material and growth conditions

A total of ten local rice varieties used in this study were kindly provided by department of Plant Breeding and Genetics, The University of Agriculture Peshawar, Pakistan. These varieties included Jajai-77, Pakhal, Shadab-31, TN-1, Shaheen Basmati, Sarshar, RI-DR-92, JP-5, NIR-9, and Sada Hayat. Seeds of these varieties were surface sterilized, grown in labeled pots and kept in green house located at Institute for Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Pakistan.

DNA isolation

DNA was extracted according to the protocol as described by EDWARDS *et al*. (1991). Briefly, about 2-3 healthy leaves from every plant were ground to fine powder and were transferred to a sterile micro tube. 400 µL of DNA extraction buffer (200 mM Tris-Hcl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) were added followed by stirring for 5s and centrifugation at 13000 rpm for 5 minutes. Supernatant (300 mL) was transferred to new sterile micro tube and 300 µL of iso-propanol was added. The tubes were inverted gently for 2 minutes at room temperature and centrifuged at 13000 rpm for 10 minutes. The pellet was washed with 70% ethanol after disposing the supernatant. The ethanol was discarded and the pellet was re-dissolved in 50 µL of nuclease free water and was stored at -20 °C.

Gel electrophoresis

The extracted DNA (12µL) mixed with 6x loading dye (Fermentas) was loaded on pre stained 1% agarose gel in 0.5X TAE buffer to confirm the presence of nucleic acids.

Quantification of extracted DNA

The extracted DNA samples were quantified through DyNA QuantTM200 flourometer and were diluted to 20ng/µL with the help of nuclease free water. The diluted genomic DNA was then stored at -20 °C for later use.

Primer selection

A total of 24 random Amplified Polymorphic DNA (RAPDs) designed by the gene Link (New York, USA) were checked as single primers to identify the most promising detectable polymorphisms. Out of these 24 RAPD markers, only nine were able to detect the polymorphisms between different cultivars and production of the reliable and scorable banding patterns in rice cultivars while 15 markers failed to produce reproducible results. Sequence information for the primers is given in Table 1.

Table 1. List of RAPD primers that efficiently detect polymorphism between different cultivars.

Primer name	Sequence	TM (ºC)	Mol.Wt (Da)
GL Decamer G-14	GGATGAGACC	3077.02	29.5
GL DecamerA-04	AATCGGGCTG	3068.02	29.5
GL DecamerD-08	GTGTGCCCCA	3003.99	33.6
GL DecamerA-05	AGGGGTCTTG	3099.04	29.5
GL DecamerD-09	CTCTGGAGAC	3028	29.5
GL DecamerD-19	CTGGGGACTT	3059.02	29.5
GL DecamerC-12	TGTCATCCCC	2938.96	29.5
GL DecamerE-04	GTGACATGCC	3028	29.5
GL Decamer A-03	AGTCAGCCAC	2996.98	29.5

PCR amplification

The Polymerase Chain Reaction (PCR) was performed following the previous protocol with minor modifications (ALHANI and WILKINSON, 2000). The reactions were carried out in a 200µL PCR tubes containing 25µL reaction mixture containing 20ng template DNA, 0.4 µM of 10-mer primer, 2.5 units of taq polymerase, 12.5 µL of PCR master mix (Fermentas) and nuclease free water. Amplifications were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) with thermal profile: initial denaturation at 94ºC for 5min followed by 35 cycles of denaturation at 94ºC for 50s, annealing at 29ºC for 45 s and extension at 72 ºC for 1min, with final extension at 72ºC for 10min.

Electrophoresis and documentation

PCR amplified products were run on 2% agarose gel, prepared in TAE buffer (1X) pre-stained with 10 µg/mL ethidium bromide used as a florescent dye and electrophoresed at 110 V for 1 hour and 30 min. The gel was visualized by using UV tech gel documentation system using1000bp of DNA ladder as marker.

Data analyses

The statistical analysis of Random Amplified Polymorphic DNA (RAPD) was performed and all the countable bands were considered as single locus/allele. The loci were scored as present or absent. Bi-variate 1-0 data matrix was generated and the genetic dissimilarities were calculated using UPGMA method (NEI and LI, 1979). $GD = 1 - dxy/dx + dy - dxy$ Where GD= Genetic distance between two genotypes, *dxy*= total number of common loci (bands) in two genotypes, *dx*= Total number of loci (bands) in genotype 1, and *dy*= Total number of loci (bands)

in genotype 2. A dendrogram was constructed on the basis of bivariate data matrix of all the nine RAPD primers using GENETYX-WIN program.

RESULTS AND DISCUSSION

Genetic diversity is vital for the adaptation of rice on diverse agroecological origins. Assessment of genetic diversity is primal factor for characterization of rice varieties, efficient breeding and most importantly in the germplasm preservation (LAXUMAN *et al*., 2011; RABBANI *et al*., 2008). Classical breeding affects the genetic diversity by selection of combination out comes from desirable allele frequencies which lead to favorable effects but loss of diversity. It is important to study the genetic polymorphism between the varieties prior to crossing them. Diversity leads to crop improvement, therefore crossing the highly diverse varieties can have good impact on the outcome.

Numerous researchers have previously demonstrated molecular diversity in rice using molecular marker tools (RAVI *et al*., 2003; SAKER *et al*., 2012; RABBANI *et al*., 2008; UPADHYAY *et al*., 2011; SIDDIQUE *et al*., 2024) and has been used for the identification, protection and parentage determination of plant hybrids. In present study, RAPD markers showed a good polymorphic data in the selected rice varieties. Therefore, these RAPD can be used for genetic diversity analysis. These finding were found similar as given by (KANAWAPEE *et al*. 2011; CHAKRABORTY *et al*. 2013; KARANDE *et al*., 2017). In our study the results of genetic diversity studies revealed different level of genetic polymorphism among *Oryza sativa* cultivars for different RAPD primers. It was observed that different primers showed variable genetic distances at DNA level, which might be due to similarity of sequence of primers with the complementary sequence in *Oryza sativa* genome. The PCR analysis of 10 rice varieties taken in this study, with 09 polymorphic random markers generated 444 scorable bands. Among RAPD markers, Genelink C-12 produced maximum number of bands (76 in all varieties) (Figure 1, Table 2) followed by GL D-08 (72) and GL A-04 and GL D-09 (62). While RAPD marker GL G-14 generated minimum number of bands (26) in the genomic pool. Genetic distance estimated in percent during present study varied from 28 to 64 (Table 3). Maximum genetic distance was found between TN-1 and Pakhal, RI-DR-92 and Sarshar (64%), followed by JP-5 and Sada Hayat (62%), Sarshar and Sada Hayat (62%), Sarshar and Shadab-31 (62%). Minimum genetic distance (28%) was observed between Jajai-77 and JP-5. In this study the level of polymorphism observed was parallel as observed by other researchers using RAPD markers. In the earlier studies KANAWAPEE *et al*. 2011, observed 68.94% polymorphism using 20 RAPD primers among 30 salinity tolerant cultivars. All the nine primers were able to produce a total of 444 amplicons with an average of 49. 33 amplicon per primer (Table 2). The number of polymorphic amplicons generated were 356 and they show 80.18 % polymorphism with an average number of polymorphic bands per primer were 39.55 (Table 2). Different primers resulted in varying degrees of polymorphism across the various genotypes. The number of DNA amplified amplicon per primer ranged from 26 to 76 (Table 2). The primer GL A-05 was the most informative primer that produced 32 polymorphic amplicons and showed 100 percent polymorphism while the primer GL A-04 was observed to be the least informative primer that shows only 68 % polymorphic bands (Table 2). KARANDE *et al*., 2017 observed high level (87.25 %) of genetic

polymorphism in 12 rice varieties using 4 RAPD primers. PERVAIZ *et al*., 2010 identified similar 80 % polymorphism among 75 Pakistan rice accessions utilizing 24 RAPD primers. For every RAPD primer, remarkable genetic variability was observed in case of RAPD primer GL A-05, whereas no polymorphism was observed by primer GL G-14. According to RAGHUNATHACHARI *et al*., 2000, the primers with 60-70% GC content gave 95.1% genetic variability. In the current study, the primers with the same GC content showed 64% polymorphism.

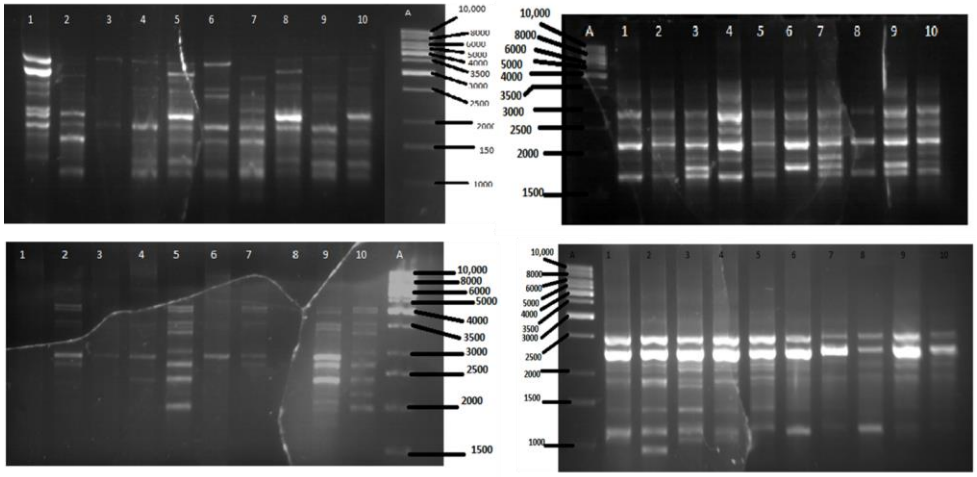

Figure 1. PCR profile of 10 genotypes of rice using RAPD primer GL C-12, GL D-08, GL D-19, GL A-04 (A. 1Kb ladder, 1. Jajai-77, 2. Pakhal, 3. Shadab-31, 4. TN-1, 5. Shaheen Basmati, 6. Sarshar, 7. RI-DR-92, 8. JP-5, 9. NIR-9, 10. Sada Hayat.)

Table 2. List of RAPD primers and polymorphic amplicons generated.

S. No.	Primer code	Total No of amplicons	No. of polymorphic amplicons	Percent polymorphism
1	GL G-14	26	0	0
2	GL A-04	62	42	68
3	GL D-08	72	62	86
4	GL A-05	32	32	100
5	GL D-09	62	60	96
6	GL D-19	35	34	97
7	GL C-12	76	66	86
8	GL E-04	39	30	76
9	GL A-03	40	30	75
	Total	444	356	
	Average	49.33	39.56	80.18

Table 3. Average genetic distances (in percentage) among rice varieties by using nine RAPD primer

	1	2	3	4	5	6	7	8	9
2	36								
3	42	50							
4	41	64	55						
5	33	57	56	53					
6	36	55	62	36	56				
7	35	46	45	49	51	51			
8	28	58	57	54	56	64	55		
9	36	46	54	41	54	49	59	58	
0	31	55	56	57	48	62	54	62	32

1. Jajai-77, 2. Pakhal, 3. Shadab-31, 4. TN-1, 5. Shaheen Basmati, 6. Sarshar, 7. RI-DR-92, 8. JP-5, 9. NIR-9, 10. Sada Hayat

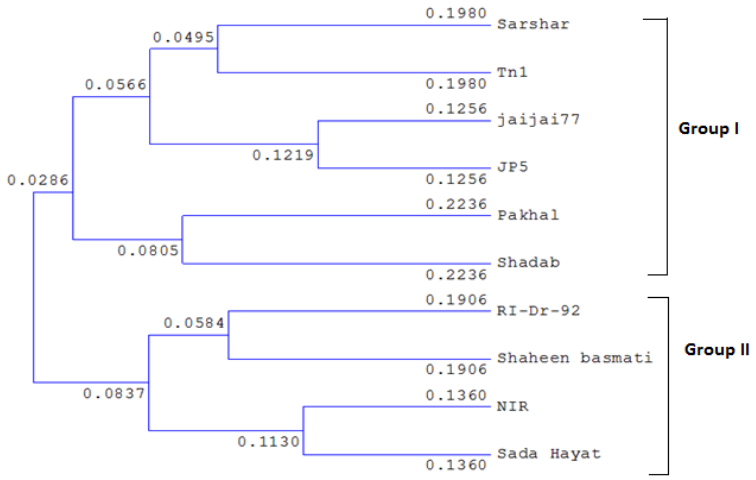

Figure 2.Dendrogram constructed for selected *Oryza sativa* varieties using unweight pair group method of arithmetic means (UPGMA).

The 1-0 bivariate data matrix for ten rice varieties based on the data of nine RAPD primers using UPGMA method was used to construct a dendrogram (Figure 2). Cluster analysis grouped six rice varieties i.e. Sarshar, TN-1, Jajai-77, JP-5, Pakhal and Shadab into one group, indicating the similarities between these varieties while the four rice varieties RI-DR-92, Shaheen Basmari, NIR-9 and Sada Hayat were found to be different from the six varieties grouped together. The cluster analysis placed RI-DR-92 and Sarshar in the different groups

confirms the maximum genetic distance between the two varieties as shown by the genetic distance (64%) estimated in percent. Similarly, the varieties JP-5 and Sada Hayat are also placed in different groups confirms their dissimilarity as shown by the estimated genetic distance in percent (62%). The dissimilarity between the two groups was recorded as 33.27%. The genetic distance observed in the rice varieties is similar both in the dendrogram and dissimilarity matrix. Nevertheless, it is noted that minimum genetic dissimilarity is found between Jajai-77 and JP-5 (28%) in the dissimilarity matrix are grouped together in the dendrogram as well. Rice cultivars with highest genetic diversity i.e. RI-DR-92 and Sarshar and JP-5 and Sada Hayat can be crossed to broaden the genetic base of *Oryza sativa*.

CONCLUSION

In this study, RAPD analysis has been found to be a valuable DNA marker system to evaluate genetic diversity for rice genotypes. Our results have demonstrated that Pakistani rice varieties possess considerable variation in their genomes. Our experiments pass on the information about genetic similarity prevails among the rice cultivars, which will be helpful to avoid any chance of elite germplasm becoming genetically uniform. Based on this study, we can recommend that the rice cultivar with wider genetic distance can be used as parents to exploit heterosis in future rice breeding programs for crop improvement. It is also suggested that more informative primers can be converted to sequence tagged sites (STS) and sequence characterized amplified regions (SCAR) for the amplification of specific alleles which can aid further in rice genome analysis.

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ANALIZA GENETSKOG DIVERZITETA PAKISTANSKIH ODABRANIH LOKALNIH SORTI PIRINČA KORIŠĆENJEM RAPD MARKERA

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Izvod

Procena genetske raznovrsnosti je veoma važna za unapređenje mnogih vrsta useva, uključujući pirinač. Studija je sprovedena radi procene genetske raznovrsnosti koja preovlađuje među deset komercijalno uzgajanih sorti pirinča u Pakistanu koristeći devet markera nasumično amplifikovane polimorfne DNK (RAPD). Svih devet prajmera je bilo u stanju da proizvede ukupno 444 amplikona sa prosekom od 49,33 amplikona po prajmeru. Ukupno je generisano 356 polimorfnih amplikona koji su pokazali 80,18% polimorfizma sa prosečnim brojem od 39,55 polimorfnih traka po prajmeru. RAPD marker, GL C-12, proizveo je maksimalan broj traka (76 kod svih sorti), dok je GL G-14 generisao minimalan broj traka (26) u genomskom pulu. Visok nivo genetskog polimorfizma na nivou DNK primećen je kod deset sorti *Oryza sativa* sa prosečnom genetskom udaljenošću u rasponu od 28% do 64% na osnovu matrice prosečnih koeficijenata različitosti nakon UPGMA. Dendrogram konstruisan korišćenjem informacija o matrici prosečnih koeficijenata različitosti svih deset sorti na osnovu podataka devet RAPD prajmera svrstao je sorte u dve kategorije. Klaster analiza je grupisala šest sorti pirinča, tj. Sarshar, TN-1, Jajai-77, JP-5, Pakhal i Shadab, u jednu grupu, što ukazuje na sličnosti između ovih sorti, dok su četiri sorte pirinča RI-DR-92, Shaheen Basmari, NIR-9 i Sada Hayat bile različite od šest sorti grupisanih zajedno. Klaster analiza koja je smestila RI-DR-92 i Sarshar u različite grupe potvrđuje maksimalnu genetsku udaljenost između dve sorte, što je prikazano genetskom udaljenošću (64%) procenjenom u procentima. Dendrogram je pokazao da su RI-DR-92 i Sarshar i JP-5 i Sada Hayat veoma udaljeni jedna od druge i da se mogu ukrštati kako bi se proširila genetska baza *Oryza sativa*. Pored toga, informativniji prajmeri mogu se konvertovati u mesta označena sekvencama (STS) i amplifikovane regione karakterisane sekvencama (SCAR) za amplifikaciju specifičnih alela, što može dalje pomoći u analizi genoma pirinča.

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