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VARIETAL IDENTIFICATION OF RICE (*ORYZA SATIVA* (L.) GENOTYPES BY RAPD MARKERS.

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Abstract

Varietal identification of rice by RAPD markers allows precise, objective and rapid method. The materials which consists of 20 rice genotypes *viz.*,TKM-1, TKM-6, TKM-9, TKM-11, TKM-12, TKM-14, AD-06207, AD-08142, CB-05031, CO-43, CO-49, HKR-081, HUR-1204, RNR-2448, RNR-2836, AD-07312, ADT-36, ADT-37, ADT-45and ADT-48 were subjected to molecular characterization using Random Amplified Polymorphic DNA (RAPD) markers. Among 20 rice genotypes, 10 RAPD primers were used to check polymorphism in which nine primers were polymorphic. 10 RAPD primers were screened and ten primers possessed scorable bands. A total of 422 RAPD markers were amplified. The size of RAPD amplicons was in between 200 bp in RPI-10 to 1100bp in RPI-8. The RAPD primer RPI-7 generated the maximum 70 markers. The RAPD primer RPI-3 generated monomorphic profile. UPGMA was performed using Jaccard's similarity coefficient matrices calculated from ten RAPD markers to generate a dendrogram for 20 rice genotypes. The RAPD primer RPI-1 could identify seven genotypes of rice (TKM-6, TKM-9, TKM-11, AD-08142, HKR-081, AD-07312 and ADT-48). The RAPD primer RPI-2 could identify three genotypes of rice (TKM-11, TKM-12 and AD-08142). The RAPD primer RPI-4 could identify three genotypes (CO-43, RNR-2448, RNR-2836). RPI-5 could identify the genotypes (TKM-1, TKM-11, TKM-12, AD-06207, ADT-37, ADT-48), RPI-6 could identify the genotypes of TKM-12, TKM-14, CB-05031, ADT-36 and RPI-7 could identify the genotypes TKM-9, TKM-12, CO-49, RNR-2836 and ADT-48. The RAPD primer RPI-8 could identify the genotype (ADT-37). RPI-9 could identify the genotypes (TKM-1, TKM-9, TKM-12, TKM-14, AD-08142, CB-05031, HKR-081, HUR-1204) and RPI-10 could identify the genotypes TKM-11, AD-06207 and CO-49.

Key words: Varietal identification, RAPD, Polymorphic Information Content (PIC), Unweighted Pair-Group Method Arithmetic mean (UPGMA).

Introduction

Rice (*Oryza sativa* L.) (2n=24) belonging to the family *Poaceae*, subfamily *Oryzoideae* is the staple food for one third of the world population and occupies almost one-fifth of the total area covered under cereals. It is grown under diverse environmental conditions and over wide geographical range. Most of the world's rice is cultivated and consumed in Asia, which constitutes more than half of the world's population. The population of rice consumers increasing at the rate of 1.8 per cent annually and the annual rice production of 643 million tonnes in 2006 must be increased to 850 million tonnes by 2025. This projected production must be achieved in the background of increasing water scarcity, decreasing arable land, biotic and abiotic stresses that rice crop faces (Collard *et al.,* 2008). The term variety is defined as an assemblage of cultivated plants, which are distinguishable by morphological, physiological, chemical and cytological characters, provided their characters are heritable, stable and distinct. Varietal development and its identification is one of the most important aspects of seed industry and seed trade. The varietal characterization and purity assessment are very important for maintenance of variety, multiplication, seed certification and seed quality control. The crop varieties can be identified by various methods like morphological, chemical, biochemical and molecular techniques. In morphological methods seed, seedling, flower and fruit characters were used for varietal characterization. The morphological differences are usually determined by a few genes and may not be representative of genetic divergence in the entire genome (Singh *et al.,* 1991).Varietal characterization using morphological characters possess several undesirable

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features like seasonal dependence, large space requirement, time consuming, tedious and environmental influence. In addition, morphological traits may not be sufficient for discrimination and identification of all extent and new varieties, warranting more precise technique. The biochemical markers (Electrophoresis of proteins and isozymes) are used for distinguishing crop varieties have been demonstrated by many workers. Though the biochemical markers are less influenced by the environmental conditions, they offer limited polymorphism and often do not allow discrimination between closely related genotypes (Ainsworth and Sharp, 1989; Aldrich *et al*., 1992). Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species. Several molecular markers *viz.*, Restriction Fragment Length Polymorphism (RFLP) (Becker *et al*., 1995; Paran and Michel more, 1993), Random Amplified polymorphic DNA(RAPD) (Tingey and Deltufo, 1993; Williams *et al.*, 1990), Simple Sequence Repeats (SSRs) (Levinson and Gutman, 1987), Inter Simple Sequence Repeat (ISSR) (Albani and Wilkinson, 1998; Blair *et al*., 1999), Ammplified Fragment Length Polymorphism (AFLP) (Mackill *et al*., 1996; Thomas *et al*., 1995; Vos *et al.*, 1995; Zhu *et al.*, 1998) and Single Nucleotide Polymorphisms (SNP's) (Vieux *et al*., 2002) are presently available to assess the variability and diversity at molecular level (Joshi *et al*., 2000). DNA marker is a new approach based on DNA polymorphism among tested genotypes and thus applicable to biological research. It offers many advantages over other categories of markers such as morphological, cytological or biochemical markers. Among all the DNA markers currently available, microsatellites are considered to be the marker of choice for varietal identification, because of their co-dominant segregation and their ability to detect large number of discrete alleles repeatedly, accurately and efficiently (Olufowote *et al*., 1997). This present study will emphasize on to characterize and identify of rice genotypes by Random Amplified polymorphic DNA (RAPDs) markers.

Materials and Methods

The materials used in the present study consisted of 20 rice genotypes (TKM-1, TKM-6, TKM-9, TKM-11, TKM-12, TKM-14, AD-06207, AD-08142, CB-05031, CO-43, CO-49, HKR-081, HUR-1204, RNR-2448, RNR-2836, AD-07312, ADT-36, ADT-37, ADT-45 and ADT-48). The pure seeds of all the genotypes were collected from different Paddy Breeding Station. All the 20 rice genotypes were subjected to molecular characterization using Random Amplified polymorphic DNA (RAPDs) markers. All the 20 genotypes were grown in raised nursery bed, Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University. Seedlings of 20-25 days old were selected for DNA extraction.

DNA extraction

Extraction of total genomic DNA was carried out by using the method described by Doyle and Doyle (1987) with some modifications. 12.14g of Trizma base (MW=121.14) was dissolved in 75ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl in a fume hood. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4°C. 29.22g of sodium chloride (NaCl, MW = 58.44) was dissolved slowly (not at once) in 75 ml of distilled water. The total volume of the solution was adjusted to 100 ml with distilled water. The solution was then heated by oven for 15 seconds and stirred thoroughly on a magnetic stirrer to dissolve NaCl. It was then sterilized by autoclaving and stored at 4°C. β-Mercaptoethanol (Himedia, Mumbai) was obtained as a 14.4 M solution from company and it was stored in a dark bottle at room temperature. 10mg RNase-A (Himedia, Mumbai) was dissolved in 1 ml of de-ionized distilled water and stored in -20°C.The crystal phenol was melted in a water bath at 65°C for 30 minutes. Melted phenol (100 ml) was added to same volume of Tris-HCl (pH 8.0). It was mixed initially for at least 10 minutes with a magnetic stirrer and then kept in rest for 5 minutes. At this stage, two distinct phases were visible, colorless upper phase and colorful lower phase. With the help of a dropper, the upper phase was removed as much as possible. The same procedure was repeated until the pH of the lower phase rose up to 7.8. Repetition for several times was needed. In this experiment, six times repetitions were done which required about 3.5 hours for obtaining the pH 7.75. After saturation, the phenol became the half of the initial volume. 50 ml of Phenol, 48 ml of Chloroform and 2 ml of Isoamyl alcohol were added and mixed properly by vortex mixture under a fume hood. The solution was then stored at 4°C. The solution was shaken well before each use. 30 ml double distilled water (ddH_2O) was added in 70 ml absolute ethanol. 1 ml of 1 M Tris-HCl was added to 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4°C. 40.824g of sodium acetate was mixed with 70 ml of ddH_2O, adjusted the final volume to 100 ml with ddH_2O and sterilized by autoclaving. To prepare extraction buffer the following components with

proper concentrations were used. For the economic use of chemicals, different volumes of solutions were prepared as in the tabular form Table 1.

10 ml of 1 M Tris-HCl (pH 8.0) was taken in a 250 ml conical flask, 28 ml 5 M NaCl was added to it 4 ml of 0.5M EDTA (pH 8.0) was taken in the conical flask. The mixture was then autoclaved, after autoclaving, 2g CTAB was added and stirred very carefully. 700 ìl â-Mercaptoethanol was added prior use and mixed by glass rod under fume hood pH of all solutions were adjusted to 5 with HCl and make up to 100 ml by adding sterile de-ionized distilled water.

RAPD analysis

A total of ten RAPD primers synthesized by Sigma Aldrich Chemical Pvt. Ltd. Bangalore, were used for PCR amplification. The details of RAPD primer used for PCR amplification are given in Table 2.

Amplification of genomic DNA using RAPD primers through polymerase chain reaction (PCR)

The genomic DNA of the different rice genotypes isolated as described earlier were subjected to PCR amplification in thermal cycler (Eppendorf, USA) the reaction volume of 15 µl containing 2 µl of genomic DNA 1X assay buffer, 200 mM of deoxy ribo nucleotides, 2 µM of $Mgcl_2$, 0.2 µM of primer, 1 unit of Tag DNA polymerase and 6.6 µl of sterile water. The PCR profile adopted was: (i) initial denaturation at 95°C for 2 minutes, followed by (ii) 34 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C and extension at 72°C for 1 minute and 30 seconds and (iii) final extension at 72°C for 10 minutes and at 4°C for cooling. Annealing temperature was standardized for each primer and adopted for all the primers used in the study as identified by their specific temperature requirement.

Calculation of PIC value

Polymorphic Information Content (PIC) values were calculated for each of the RAPD loci using the formula developed by Roldan-Ruiz *et al*. (2000).

$$PIC = 2f_i (1 - f_i)$$

Where,

f_i is frequency of marker bands which were present and 1-fi frequency of markers bands which were absent.

Cluster analysis

The scoring data in the form of binary values was used for the construction of dendrogram. The genetic associations between varieties were evaluated by calculating the Dice's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers (Dice, 1945). Similarity matrix was generated using the SIMQUAL programme of NTSYS-pc software, version 2.02 (Rohlf, 1998). The similarity coefficients were used for cluster analysis and dendrogram was constructed by the Unweighted Pair-Group Method Arithmetic mean (UPGMA) (Sneath and Sokal, 1973).

Results

The results of the experiment including 20 rice genotypes using molecular markers Random Amplified Polymorphic DNA (RAPD) are presented as follows.

RPI-1

The genotypes CB-05031 and HUR-1204 did not show any band with this primer. The genotypes AD-08142 possessed eight bands. The genotypes TKM-6 and AD-07312 possessed five bands each. The genotypes TKM-14 and RNR-2836 possessed four bands each. The genotypes TKM-1, TKM-9, TKM-11, TKM-12, CO-49, RNR-2448, ADT-36 and ADT-48 possessed three bands each. The genotypes AD-06207, CO-43, HKR-081 and ADT-37 possessed two bands each. The genotype ADT-45 possessed single band with this primer Fig. 1.

RPI-2

The genotypes TKM-9, AD-06207, CB-05031, HUR-1204, RNR-2448 and ADT-45 did not show any band with this primer**.** The genotypes TKM-11, TKM-12, AD-08142, CO-43, CO-49, HKR-081, RNR-2836, ADT-36, ADT-37 and ADT-48 possessed two bands each. The genotypes TKM-1, TKM-6, TKM-14 and AD-07312 possessed single band each with this primer Fig. 2.

RPI-3

All the genotypes showed single band with this primer. The primer shows monomorphic banding pattern and the band is placed in 200 bp Fig. 3.

RPI-4

The genotypes TKM-11, TKM-14 AD-08142, CB-05031, AD-07312, ADT-37, ADT-45 and CO-49 did not show any band with this primer**.** The genotypes CO-43, HKR-081 and ADT-48 possessed three bands each. The genotypes TKM-1, TKM-6, TKM-12, AD-06207, HUR-1204, RNR-2448 and ADT-36 possessed two bands each. The genotypes TKM-9 and RNR-2836 possessed single band each with this primer Fig. 4.

RPI-5

The genotypes TKM-14, AD-08142, ADT-36 and ADT-45 did not show any band with this primer. The genotype AD-06207 possessed six bands. The genotypes TKM-11, CO-43, CO-49, HKR-081, HUR-1204, RNR-

2448, RNR-2836 AD-07312 and ADT-48 possessed five bands each. The genotypes TKM-1, TKM-6 and ADT-37 possessed four bands each. The genotype TKM-12 possessed three bands. The genotypes CB-05031 possessed two bands. The genotype TKM-9 possessed single band with this primer Fig. 5.

RPI-6

The genotypes TKM-9, TKM-11, CO-43, HUR-1204 and ADT-37 did not show any band with this primer. The genotype RNR-2836 possessed five bands. The genotypes ADT-45 and ADT-48 possessed four bands each. The genotypes TKM-14, CB-05031, CO-49, HKR-081, RNR-2448 and ADT-36 possessed three bands. The genotypes TKM-1, TKM-6, TKM-12, AD-06207 and AD-08142 possessed two bands each. The genotype AD-07312 possessed single band with this primer Fig. 6.

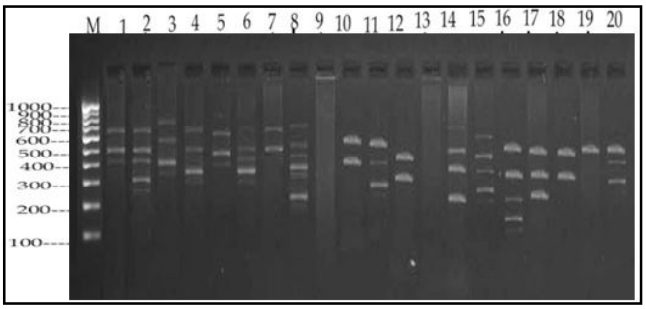

Fig. 1: RAPD Analysis with primer RPI–1 (AAAGCTGCGG) on 20 genotypes of rice.

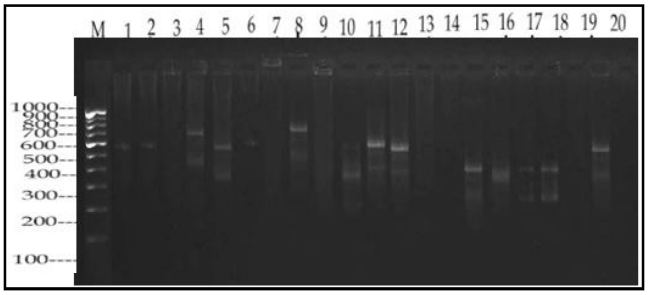

Fig. 2: RAPD Analysis with primer RPI–2 (AACGCGTCGG) on 20 genotypes of rice.

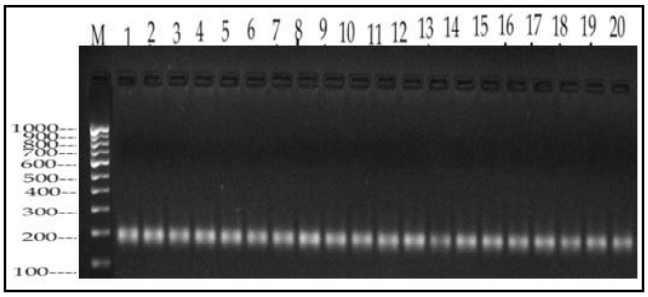

Fig. 3: RAPD Analysis with primer RPI–3 (AAGCGACCTG) on 20 genotypes of rice.

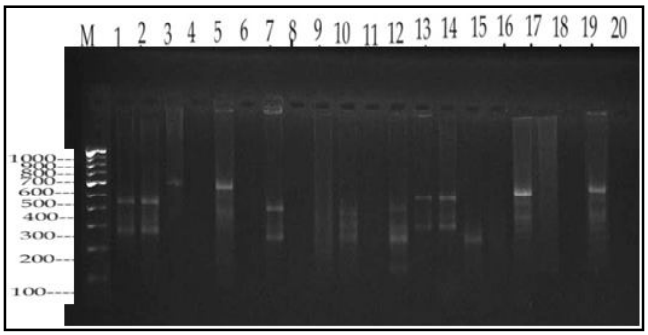

Fig. 4: RAPD Analysis with primer RPI–4 (AATCGCGCTG) on 20 genotypes of rice.

RPI-7

The genotype TKM-12 possessed six bands. The genotypes TKM-9, HUR-1204 and RNR-2448 possessed

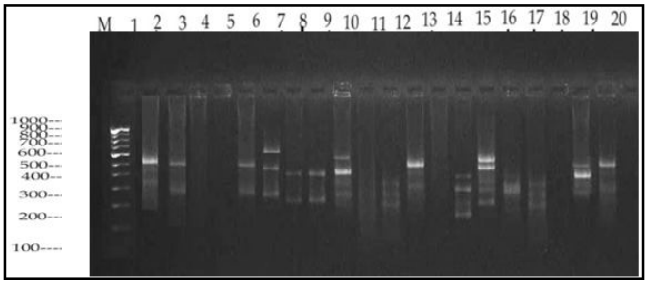

Fig. 6: RAPD Analysis with primer RPI–6 (ACACACGCTG) on 20 genotypes of rice.

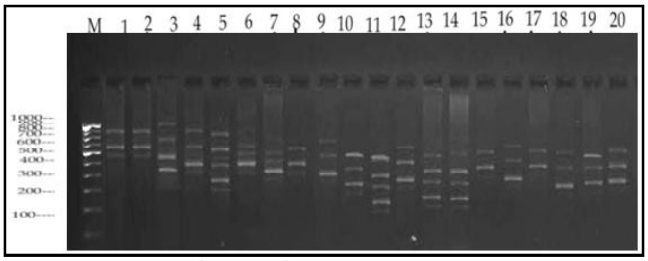

Fig. 7: RAPD Analysis with primer RPI–7 (ACATCGCCCA) on 20 genotypes of rice.

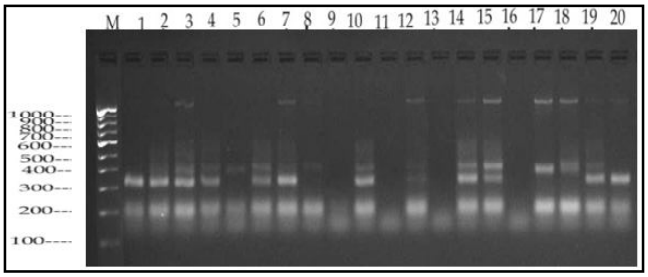

Fig. 8: RAPD Analysis with primer RPI-8 (ACCACCCACC) on 20 genotypes of rice.

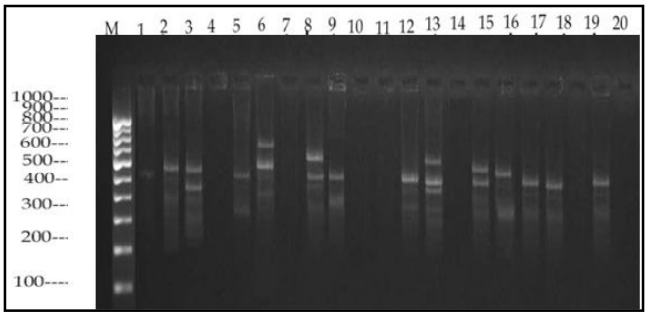

Fig. 9: RAPD Analysis with primer RPI–9 (ACCGCCTATG) on 20 genotypes of rice.

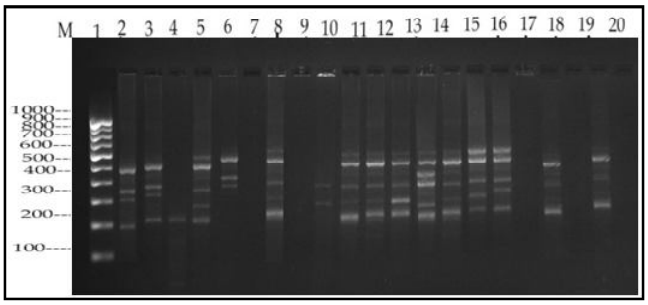

Fig. 5: RAPD Analysis with primer RPI–5 (AATCGGGCTG) on 20 genotypes of rice.

Table 1: Volume of solutions to prepare extraction buffer.

Chemicals	Mole cular weight	Stock concen tration	Working concen tration	Working volume	
				100 ml	1000 ml
CTAB	---	---	2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA	372.24	0.5 M	20 mM	4 ml	40ml
Tribase (pH 8)	121.1	1.0 M	100 mM	10 ml	100 ml
β-Mercaptoethanol	---	14.4 M	100 mM	700 µl	7ml

Table 2: Details of RAPD primers used for PCR amplification.

S. No.	Primers	Sequence 5'-3'	Annealing temperature (°C)
1	RPI – 1	AAAGCTGCGG	36
2	RPI – 2	AACGCGTCGG	36
3	RPI – 3	AAGCGACCTG	36
4	RPI – 4	AATCGCGCTG	36
5	RPI – 5	AATCGGGCTG	36
6	RPI – 6	ACACACGCTG	36
7	RPI – 7	ACATCGCCCA	36
8	RPI – 8	ACCACCCACC	36
9	RPI – 9	ACCGCCTATG	36
10	RPI – 10	ACGATGAGGG	36

five bands each. The genotypes TKM-14, CB-05031 and CO-49 possessed four bands each. The genotypes TKM-1, TKM-6, TKM-11, AD-06207, AD-08142, CO-43, HKR-081, AD-07312, ADT-37, ADT-45 and ADT-48 possessed three bands each. The genotypes RNR-2836 and ADT-36 possessed two bands each Fig. 7.

RPI-8

The genotypes CB-05031, CO-49, HUR-1204 and AD-07312 did not show any band with this primer. The genotypes TKM-9, AD-06207, HKR-081, RNR-2448, RNR-2836 ADT-37 and ADT-36 possessed four bands each. The genotypes TKM-11, TKM-14, CO-43, ADT-36, and ADT-48 possessed three bands each. The genotypes TKM-1, TKM-6, and AD-08142 possessed two bands each. The genotype TKM-12 possessed single band with this primer Fig. 8.

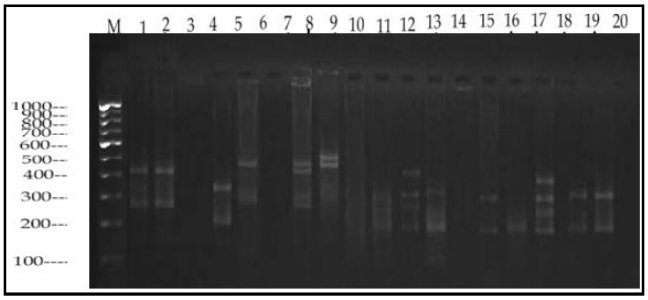

Fig. 10: RAPD Analysis with primer RPI–10 (ACGATGAGGG) on 20 genotypes of rice.

RPI-9

The genotypes TKM-11, AD-06207, CO-43, CO-49, RNR-2448 and ADT-45 did not show any band with this primer. The genotype TKM-14 possessed four bands. The genotype HUR-1204 possessed three bands. The genotypes TKM-9, TKM-12, AD-08142, CB-05031, HKR-081, RNR-1836, AD-07132, ADT-36, ADT-37 and ADT-48 possessed two bands each. The genotype TKM-1and TKM-6 possessed single band with this primer Fig. 9.

RPI-10

The genotypes TKM-9, TKM-14, CB-05031, HUR-1204, ADT-45 and ADT-48 did not show any band with this primer. The genotypes AD-06207, CO-49 and AD-07312 possessed four bands each. The genotypes CO-43 possessed three bands. The genotypes TKM-1, TKM-6, TKM-11, TKM-12, AD-08142, HKR-081, RNR-2448, ADT-36 and ADT-37 possessed two bands each. The genotype RNR-2836 possessed single band with this

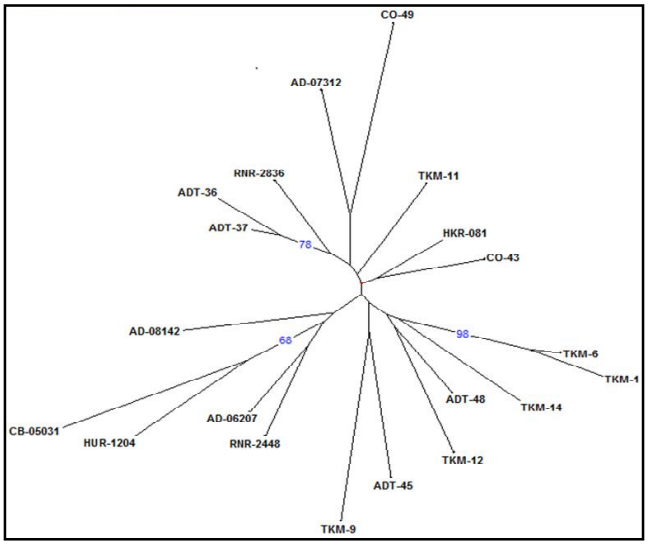

Fig. 11: Dendogram of 20 rice genotypes constructed from UPGMA cluster analysis using Jaccard's similarity coefficient based on data derived from 10 RAPD markers.

primer Fig. 10.

RAPD Analysis

The result obtained based on the analysis of 20 rice genotypes using 10 RAPD primers are furnished. The PCR amplification of template DNA produced a total 422 bands among 20 genotypes with 10 RAPD primers. A total of 422 bands were obtained using 10 RAPD primer. The number of polymorphic markers and the percentage of polymorphic among the 20 genotype analyzed were 402. The Polymorphic Information Content (PIC) for the primer ranged from 0.0792899 (RPI-6) to 0.6222 (RPI-9).

Discussion

In the present study, 10 RAPD primers were used to check polymorphism in the 20 genotypes of rice and 9 primers were found to be polymorphic. The 10 RAPD primers generated 422 markers for the assessment of genetic variability between the genotypes studied. In that 402 markers were polymorphic, 20 were monomorphic. All the genotypes showed a varying degree of genetic diversity based on their amplification profile. A high level of polymorphism was observed among the 20 genotypes studied. A similar and contradictory research findings were reported by Jain *et al.,* (2012), showed low level of polymorphism in rice. Jain *et al.,* (2012) examined four rice accessions with 10 RAPD markers which produced 124 bands and these were in the range of 150-1700 bp. Ashusingh and Sengar RS (2015) reported a similar research findings which showed a high level of polymorphism in rice. Ashusingh and Sengar RS (2015) examined 30 rice varieties using 10 RAPD markers in which PIC values varies from 0.811 (OPD-08) to 0.9925 (OPF-13) with an average of 1.8256. A contradictory research finding were reported by Sadia *et al.,* (2012), they examined 30 commercial rice varieties in which the size of the amplified fragments ranged from 200 bp (OPA-08) to 3630 bp (OPB-13). A high level of polymorphism was observed among all the genotypes in the present study. This study showed that highly divergent genetic base material under investigation, which might be due to almost different genetic makeup of genotypes. Contradictory reports on the extent of observed polymorphism in rice could be attributed to different types of genetic materials used in different studies. UPGMA was performed using Jaccard's similarity coefficient matrices calculated from 10 RAPD markers to generate a dendogram for 20 rice genotypes. It ranged from 0 to 0.2 indicated that genetic diversity among the 20 varieties. The dendogram showed the grouping pattern of eight clusters. The distribution of 20 genotypes into eight clusters was shown in Fig. 11. The 20 genotypes of rice were grouped into eight clusters. Cluster I consisted of two genotypes ADT-37 and ADT-36. Cluster II consisted of one genotype RNR-2836.Genotypes AD-07312 and CO-49 were grouped under cluster III. Cluster IV consisted of only one genotype TKM-11. Cluster V consisted of two genotypes HKR-081 and CO-43. Cluster VI consisted of seven genotypes TKM-6, TKM-1, TK M-14, ADT-48, TKM-12, ADT-45 and TKM-9. Genotype AD-08142 is grouped under Cluster VII. Cluster VIII consisted of four genotypes CB-05031, HUR-1204, AD-06207 and RNR-2448. The RAPD primer RPI-1 identified seven genotype of rice (TKM-6, TKM-9, TKM-11, AD-08142, HKR-081, AD-07312 and ADT-48). The RAPD primer RPI-2identified three genotypes of rice (TKM-11, TKM-12 and AD-08142). The RAPD primer RPI-4 identified three genotypes (CO-43, RNR-2448, RNR-2836). RPI-5 identified the genotype (TKM-1, TKM-11, TKM-12, AD-06207, ADT-37 and ADT-48), RPI-6 identified the genotypes of TKM-12, TKM-14, CB-05031, ADT-36 and RPI-7 identified the genotypes TKM-9, TKM-12, CO-49, RNR-2836 and ADT-48. The RAPD primer RPI-8 identified the genotype (ADT-37). RPI-9 identified the genotypes (TKM-1, TKM-9, TKM-12, TKM-14, AD-08142, CB-05031, HKR-081, HUR-1204) and RPI-10 identified the genotypes TKM-11, AD-06207 and CO-49.

Conclusion

The present investigation was undertaken with an objective to identify distinguishable molecular markers for the rice varieties. The RAPD markers identified were validated for their utility as molecular IDs in varietal identity. The present study also intended to assess the genetic diversity among the varieties using RAPD as well as molecular markers. Ten RAPD primers were screened and ten primers produced scorable bands. Ten RAPD primers were screened and all the ten primers produced scorable bands. Nine RAPD primers showed polymorphism. A total of 422 RAPD markers were amplified, out of which 402 were polymorphic, 20 were monomorphic. The PCR amplification of template DNA produced a total 422 bands among 20 genotypes with 10 RAPD primers. The Polymorphic Information Content (PIC) for the primer ranged from 0.792899 (RPI-6) to 0.62222 (RPI-9). The unique band possessed in RPI-8 can be used to identify ADT-37 genotype and unique band possessed by RPI-2 can identify TKM-11, TKM-12 and AD-08142 genotypes.

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