



VARIETAL IDENTIFICATION OF RICE (*ORYZA SATIVA* L.) GENOTYPES USING ISSR MARKERS

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Abstract

Varietal identification of rice by ISSR 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-45 and ADT-48 were subjected to molecular characterization using Inter Simple Sequence Repeats (ISSRs) markers. Among 20 rice genotypes, eight ISSR primers were used to check polymorphism in which five primers were polymorphic. 8 ISSR primers were screened and 5 primers possessed scorable bands. A total of 356 ISSR markers were amplified. The size of ISSR amplicons was in between 100 bp in UBC 807 to 800 bp in UBC-853. The ISSR primer UBC - 808 generated the maximum 87 markers. The ISSR primer UBC-809, UBC-824 and UBC-841 does not amplify with these genotypes. The ISSR primer UBC-808 generated highly polymorphic profile. UPGMA was performed using Jaccard's similarity coefficient matrices calculated from eight ISSR markers to generate a dendrogram for 20 rice genotypes. The ISSR primer UBC 807 could identify three genotypes of rice AD-06207, AD-08142 and CO-43. The ISSR primer UBC-808 could identify two genotypes of rice (TKM-9 and HUR-1204). The ISSR primer UBC-823 could one genotype (RNR-2448). UBC-834 could identify one genotype (AD-08142), UBC-853 could identify the genotypes (CB-05031, CO-43, RNR-2836 and AD-07312).

Key words : Varietal identification, ISSR markers, Polymorphic Information Content (PIC), UPGMA, Rice.

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

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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 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 extant 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 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 Michelmore, 1993), Random Amplified polymorphic DNA (RAPD) (Tingey and Delfino, 1993; Williams *et al.*, 1990), Simple Sequence Repeats (SSRs) (Levinson and Gutman, 1987), Inter Simple Sequence Repeats (ISSRs) (Albani and Wilkinson, 1998; Blair *et al.*, 1999), Amplified 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). Inter Simple Sequence Repeats (ISSRs) are nonfunctional and selectively neutral, linked to coding regions, so that ISSRs are likely to mark gene rich regions (Kojima *et al.*, 1998). This present study will emphasize on to characterize and identify of rice genotypes by Inter Simple Sequence Repeats (ISSRs) 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 Inter Simple Sequence Repeats (ISSRs) 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. 12.14 g of Trizma base (MW=121.14) was dissolved in 75 ml 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. 29.22 g 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. 10 mg 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 (ddH₂O) was added in 70 ml absolute ethanol. 1 ml of 1 M Tris-HCl was added to 0.2 ml (200 μ l) 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. 40.824 g of sodium acetate was mixed with 70 ml of ddH₂O, adjusted the final volume to 100 ml with ddH₂O 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.

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 0.5 M EDTA (pH 8.0) was taken in the conical flask. The mixture was then autoclaved, after autoclaving, 2 g CTAB was added and stirred very carefully 700 µl β-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.

ISSR analysis

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

Amplification of genomic DNA using ISSR 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 MgCl₂, 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 Tm requirement.

Calculation of PIC value

Polymorphic Information Content (PIC) values were calculated for each of the ISSR 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).

Table 1: Volume of solutions to prepare extraction buffer.

Chemicals	Molecular weight	Stock concentration	Working concentration	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	10ml	100ml
β-Mercaptoethanol	14.4 M	100 mM	700µl	7ml

Results

The results of the experiment including 20 rice genotypes using molecular markers Inter Simple Sequence Repeat (ISSR) are presented here under.

UBC-807

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

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

Sl. No.	ISSR-Primers	Sequences (5’-3’)
1.	UBC-807	AGAGAGAGAGAGAGAGT
2.	UBC-808	AGAGAGAGAGAGAGAGC
3.	UBC-809	AGAGAGAGAGAGAGAGG
4.	UBC-823	TCTCTCTCTCTCTCC
5	UBC-824	GAGAGAGAGAGAGAGAG
6.	UBC-834	AGAGAGAGAGAGAGAGYT
7.	UBC-841	GAGAGAGAGAGAGAAYC
8.	UBC-853	TCTCTCTCTCTCTCRT

UBC - 808

The genotypes RNR-2448 and CB-05031 did not show any band with this primer. The genotype AD-06207 possessed eight bands. TKM-9, TKM-14 and RNR-2836 possessed seven bands each. TKM-12, CO-43 and AD-07312 possessed six bands each, TKM-1, TKM-6 and AD-08142 possessed five bands each, the genotypes CO-49 and HKR-081 possessed four bands each. The genotypes TKM-11, ADT-45 and ADT-48 possessed three bands each and HUR-1204, ADT-36 and ADT-37 possessed 2 bands each (Fig. 2).

UBC - 809

All the genotypes did not show any band with this primer.

UBC - 823

The genotypes TKM-6, TKM-14, CO-43, RNR-2836 and ADT-36 did not show any band with this primer. The genotype CB-05031 and RNR-2448 had seven bands each. The genotypes AD-06207 and AD-07312 possessed six bands each, the genotypes TKM-1, TKM-9 and TKM-12 had five bands each. The genotypes TKM-11, ADT-45 and ADT-48 had four bands each. The genotype CO-49 had three bands. The genotype ADT-37 had two bands. The genotypes AD-08142, HKR-081 and HUR-1204 had single band each (Fig. 3).

UBC - 824

All the genotypes did not show any band with this primer.

UBC - 834

The genotypes TKM-11, TKM-14, RNR-2448 ADT-36 and ADT-37 did not show any bands with this primers. The genotypes TKM-1, RNR-2836 and AD-07312 had seven bands each. The genotype TKM-9 possessed six bands, the genotypes CO-43, CO-49 and HKR-081 had five bands each. The genotype TKM-6 had four bands. The genotypes AD-06207, AD-08142 and ADT-45 had three bands each. The genotypes TKM-12, CB-05031, HUR-1204 and ADT-48 had two bands each (Fig. 4).

UBC - 841

All the genotypes did not show any band with this primer.

UBC - 853

The genotypes TKM-14, AD-08142, HUR-1204, ADT-36 and ADT-45 did not show any band with this primer. The genotypes CB-05031 and ADT-48 had nine bands each, the genotype ADT-37 had seven bands. The genotypes TKM-1, TKM-11, AD-06207 and AD-07312 had five bands each. The genotypes TKM-6, TKM-12,

CO-49 and HKR-081 had four bands each. The genotype RNR-2448 had three bands. The genotypes CO-43 and RNR-2836 had single band each with this primer (Fig. 5).

ISSR ANALYSIS

The result obtained based on the analysis of 10 rice genotypes using 15 ISSR primers are furnished in (Table 4 to 13). The PCR amplification of template DNA produced a total 292 bands among 10 genotypes with 15 ISSR primers. A total of 292 bands were obtained using 15 ISSR primer with an average of 19.4 alleles per primer. The number of the alleles amplified per primer ranged from 2 to 54. The number of polymorphic markers and the percentage of polymorphic among the 10 genotype analysed were 209 and 11 per cent respectively. The Polymorphic Information Content (PIC) for the primer ranged from 0.0994 (UBC-820) to 0.8750 (UBC-841).

Discussion

In the present study, eight ISSR primers were used to check polymorphism among the 20 genotypes of rice were found to be polymorphic. The five ISSR primers generated 356 markers for the assessment of genetic variability between the genotypes studied. 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 Chowdhary *et al.* (2010), Hussain *et al.* (1989), Dongre *et al.* (2007) and Parkhiya *et al.* (2014). Chowdhary *et al.* (2010) showed low level of intra specific polymorphism in chickpea. Dongre *et al.* (2007) examined 19 ISSR primers, which generated 49 polymorphic markers out of the total 90 markers, producing 54.7 per cent polymorphism. Hussain *et al.* (2007) used 12 ISSR primers to estimate the genetic relationship among 21 cotton genotypes producing 125 amplicons with 49.6 per cent polymorphism. Parkhiya *et al.* (2014) studied genetic diversity in 15 cotton genotypes by ISSR markers and obtained 86 reproducible bands out of 54 were polymorphic with 62.7 per cent polymorphism. The present study showed that high divergence among the genotypes investigated implying the 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. The amplified ISSR fragments were in the range of 100 bp to 800 bp. The largest fragment of 800 bp were amplified by the primer UBC-807. Contradictory and similar findings were reported by Dongre *et al.* (2007). Dongre *et al.* (2007) examined 19 ISSR primers,

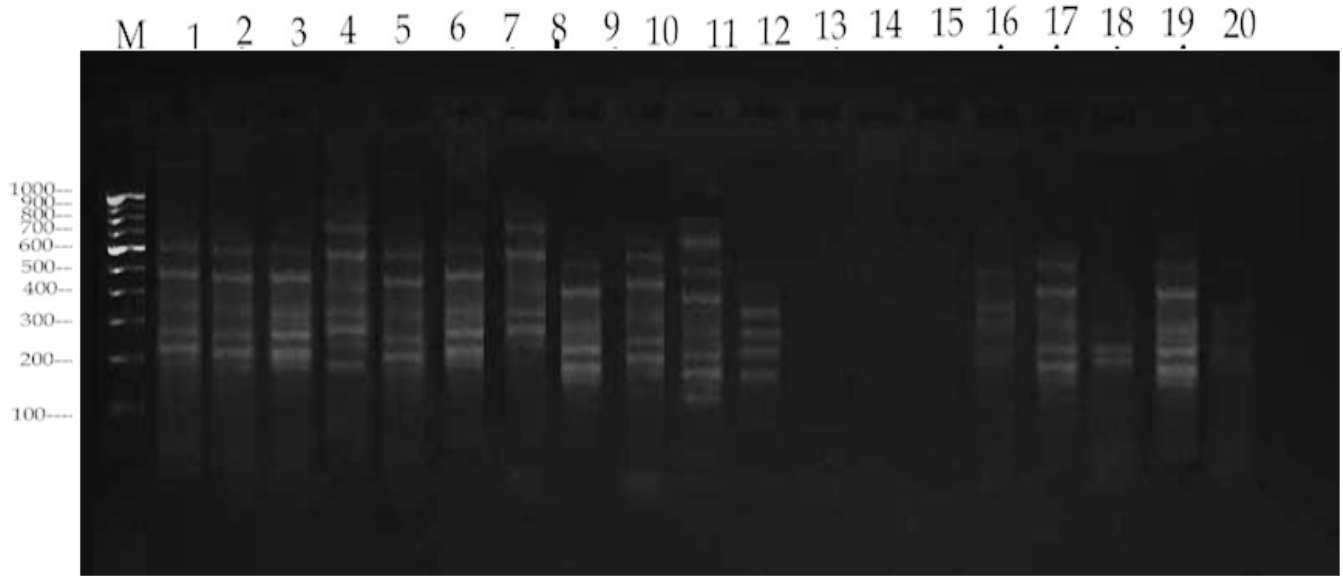


Fig. 1: ISSR Analysis with primer UBC-807 (AGA GAG AGA GAG AGA GT) on 20 genotypes of rice.

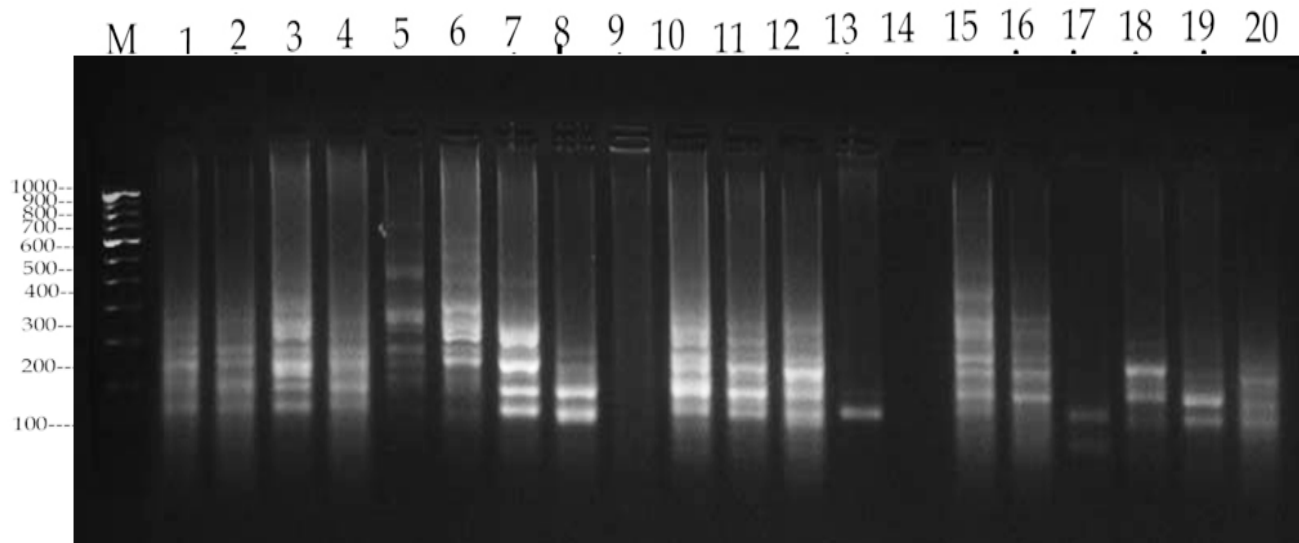


Fig. 2: ISSR Analysis with primer UBC-808 (AGAGAGAGAGAGAGAGC) on 20 genotypes of rice.

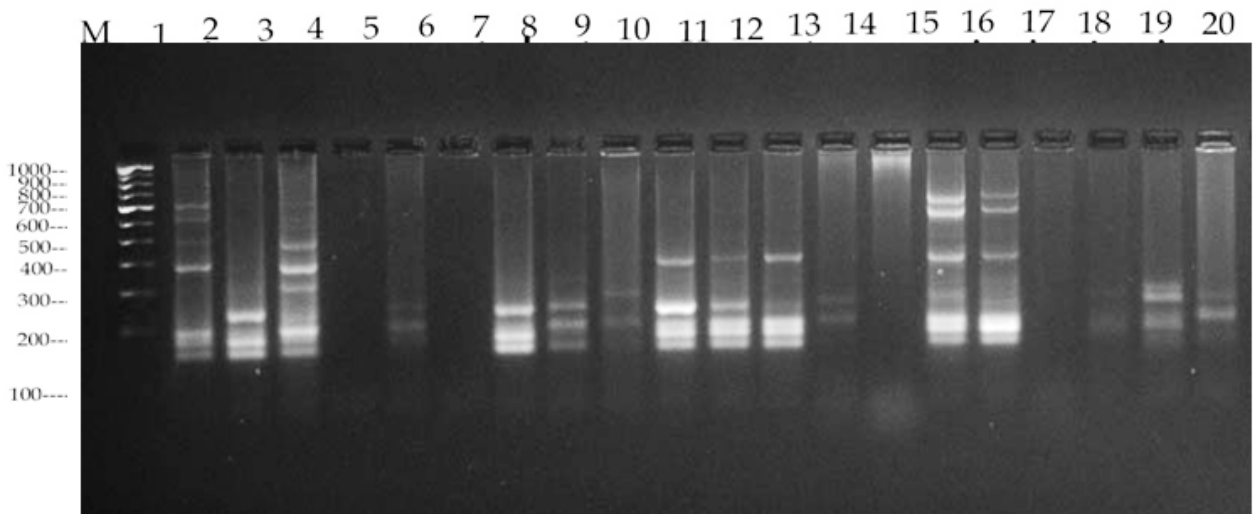


Fig. 3: ISSR Analysis with primer UBC-823 (TCTCTCTCTCTCTCC) on 20 genotypes of rice.

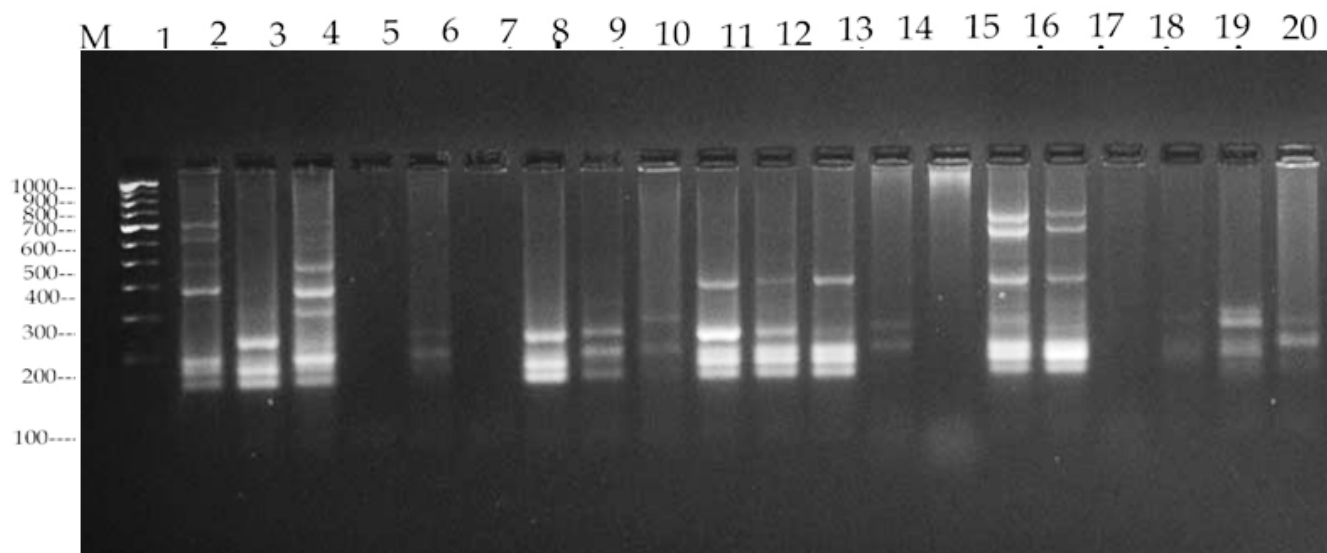


Fig. 4: ISSR Analysis with primer UBC-834 (AGAGAGAGAGAGAGAGYT) on 20 genotypes of rice.

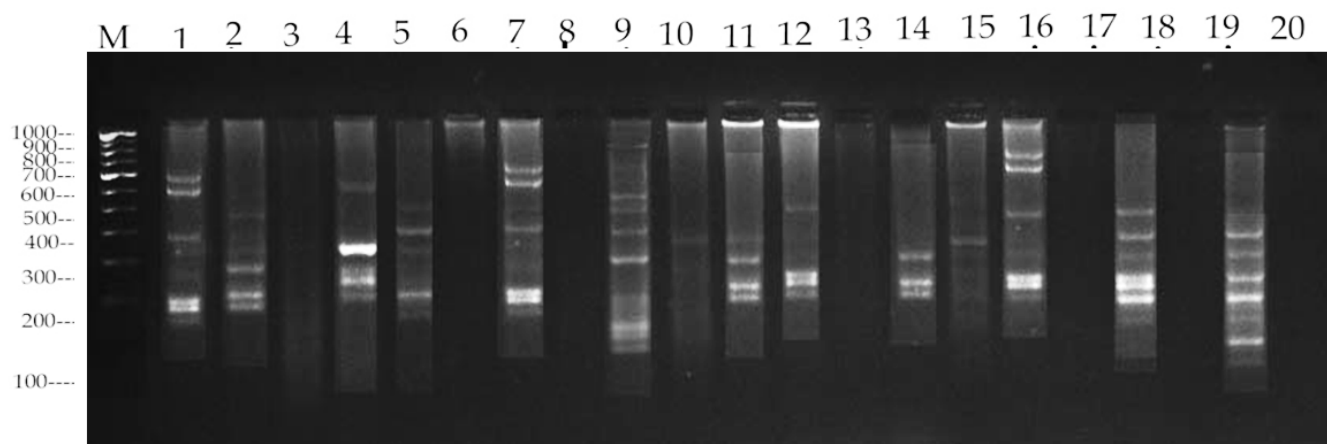
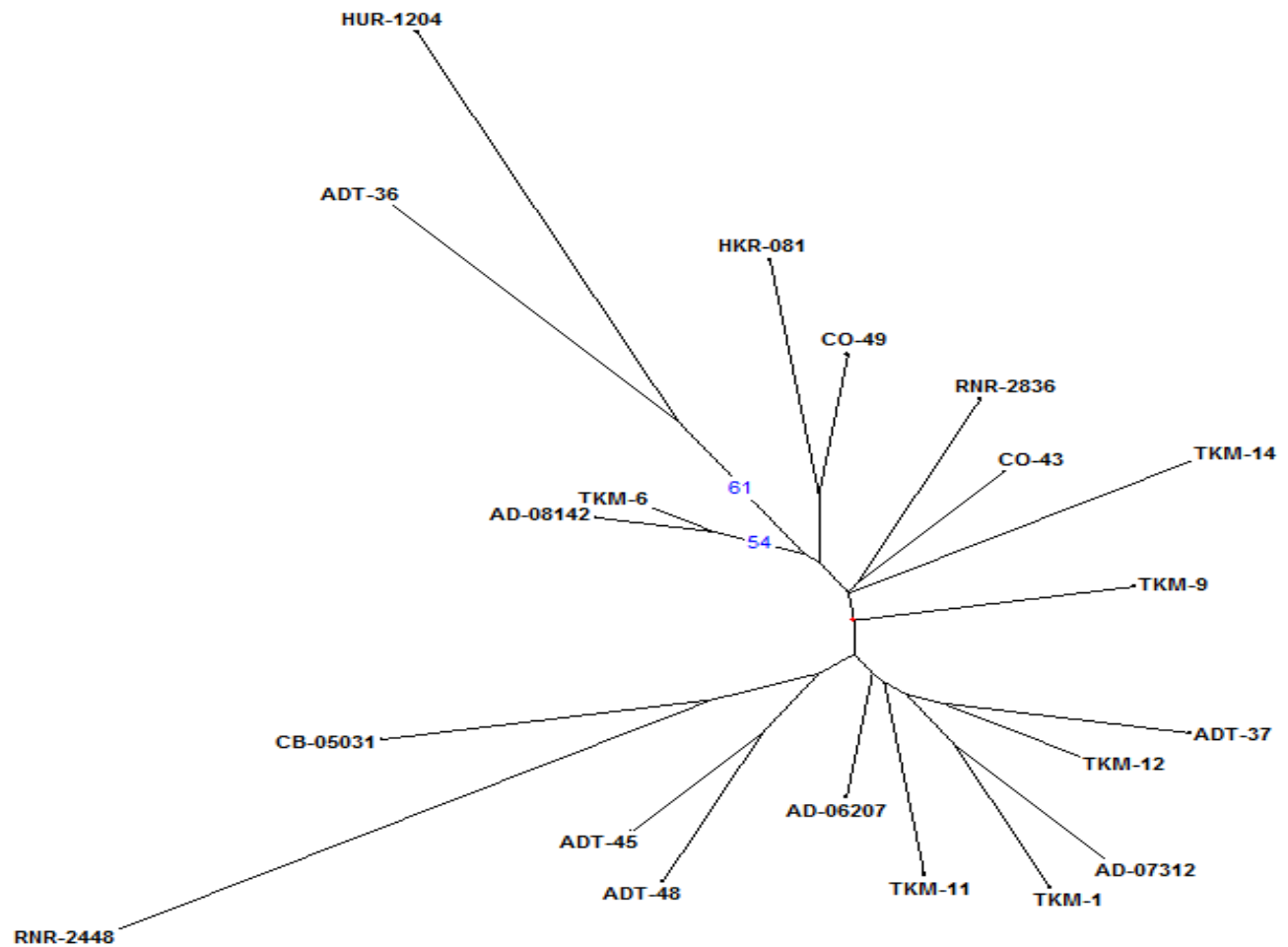


Fig. 5: ISSR Analysis with primer UBC-853 (TCTCTCTCTCTCTCRT) on 20 genotypes of rice.

M	1 kb ladder	Lane 6	TKM-14	Lane 12	HKR-081	Lane 18	ADT-37
Lane 1	TKM-1	Lane 7	AD-06207	Lane 13	HUR-1204	Lane 19	ADT-45
Lane 2	TKM-6	Lane 8	AD-08142	Lane 14	RNR-2448	Lane 20	ADT-48
Lane 3	TKM-9	Lane 9	CB-05031	Lane 15	RNR-2836		
Lane 4	TKM-11	Lane 10	CO-43	Lane 16	AD-07312		
Lane 5	TKM-12	Lane 11	CO-49	Lane 17	ADT-36		

which generated 49 polymorphic markers out of total 90 markers producing the fragment size of 1000 to 1444 bp. The sizes of fragments obtained were ranged 250 to 2600 bp. UPGMA was performed using Jaccard's similarity coefficient matrices calculated from 8 ISSR markers to generate a dendrogram for 20 rice genotypes. It ranged from 0 to 0.2 indicating the genetic diversity among the 20 varieties. The dendrogram showed the grouping pattern of eight clusters. The distribution of 20 genotypes into eight clusters. The 20 genotypes of rice were grouped into eight clusters. Cluster I consisted of two genotypes AD-08142 and TKM-6. Cluster II consist of two

genotypes ADT-36 and HUR-1204. The genotypes HKR-081 and CO-49 were grouped under cluster III. Cluster IV consisted of three genotypes RNR-2836, CO-43 and TKM-14. Cluster V consisted of one genotype TKM-9. Cluster VI consisted of six genotypes ADT-37, TKM-12, AD-07312, TKM-1, TKM-11 and AD-06207. The genotypes ADT-45 and ADT-48 were grouped under Cluster VII. Cluster VIII consisted of two genotypes RNR-2448 and CB-05031. ISSR markers were used for the unique identification of 20 genotypes of rice. The unique markers were located across all the primers that individually identified each of the genotypes. Details of



Dendrogram of 20 rice genotypes constructed from UPGMA cluster analysis using Jaccard's similarity coefficient based on data derived from 8 ISSR markers

the genotypes specific marker generated by different primers are given in Table 16. The identification is based on presence and absence of unique marker. The ISSR primer UBC 807 identified three genotypes of rice AD-06207, AD-08142 and CO-43. The ISSR primer UBC-808 could identify two genotypes of rice (TKM-9 and HUR-1204). The ISSR primer UBC-823 identified one genotype (RNR-2448). UBC-834 could identify one genotype (AD-08142), UBC-853 identified four genotypes (CB-05031, CO-43, RNR-2836 and AD-07312).

Conclusion

The present investigation was undertaken with an objective to identify distinguishable molecular markers for the rice varieties. The ISSR 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 ISSR as well as molecular markers. Eight ISSR primers were screened and five primers produced scorable bands. The Polymorphic Information Content (PIC) for the primer

ranged from 0.828277 (UBC-808) to 0.78238 (UBC-823). The 20 genotypes of rice were grouped into eight clusters. Cluster I consisted of two genotypes AD-08142 and TKM-6. Cluster II consist of two genotypes ADT-36 and HUR-1204. The genotypes HKR-081 and CO-49 were grouped under cluster III. Cluster IV consisted of three genotypes RNR-2836, CO-43 and TKM-14. Cluster V consisted of one genotype TKM-9. Cluster VI consisted of six genotypes ADT-37, TKM-12, AD-07312, TKM-1, TKM-11 and AD-06207. The genotypes ADT-45 and ADT-48 were grouped under Cluster VII. Cluster VIII consisted of two genotypes RNR-2448 and CB-05031. The ISSR primer UBC 807 identified three genotypes of rice AD-06207, AD-08142 and CO-43. The ISSR primer UBC-808 could identify two genotypes of rice (TKM-9 and HUR-1204). The ISSR primer UBC-823 identified one genotype (RNR-2448). UBC-834 could identify one genotype (AD-08142), UBC-853 identified four genotypes (CB-05031, CO-43, RNR-2836 and AD-07312).

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