Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; SP1: 2808-2812

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Studies on varietal identification of rice genotypes using ISSR markers

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Abstract
Varietal identification of rice by ISSR markers allows precise, objective and rapid method. The materials for this study consisted of 10 rice genotypes *viz.* ADT 36, ADT 37, ADT 38, ADT 39, ADT 42, CO 42, CO 43, CO 45, CO 47 and ASD 20 were subjected to molecular characterization using Inter Simple Sequence Repeats (ISSRs) markers. In 10 rice genotypes, 15 ISSR primers were used to check polymorphism in which 11 primers were found to be polymorphic. The number of ISSR markers ranged from 3 as produced by primer UBC-857 and 44 as yielded by primer UBC-820 among the ten genotypes studied. Out of the total 292 ISSR markers amplified, only 209 markers were found to be polymorphic which had resulted 71.5 per cent polymorphism. UPGMA was performed using jaccard's similarity coefficient matrices calculated from 15 ISSR markers to generate a dendrogram for 10 rice genotypes. The primer UBC-809 identified the rice genotypes ADT 36, ASD 20 and UBC-834 identified ADT 38 and CO 47 rice genotypes. The present study revealed that the ISSR primer UBC-834 identified two genotypes *viz.* ADT 38 and CO 47. The ISSR primer UBC-840 identified the genotypes ADT 3, CO 45 and UBC-841 identified the genotypes' ADT 36 and UBC 879 identified the genotypes CO 47. The ISSR primer UBC-808 identified one genotype of rice ADT 42. The ISSR primer UBC 809 identified two genotypes ADT 36 and ASD 20. The ISSR primer UBC 834 identified two genotypes ADT 38 and CO 47. UBC-840 identified the genotypes ADT 39, CO 45 and UBC-841 identified the genotype ADT 36 and UBC-879 identified the genotype CO 45.

Keywords: rice genotypes, ISSR markers.

Introduction
Rice (*Oryza sativa* L.) (2n=24) belonging to the family *Poaceae*is the staple food for one third of the world population and occupies almost one-fifth of the total area covered under cereals. 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. 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 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. 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 relationship within and among the species. 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). Hence, the present study was under taken 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 10 rice genotypes *viz.* ADT-36, ADT-37, ADT-38, ADT-39, ADT-42, CO-42, CO-43, CO-45, CO-47 and ASD-20. Genetically pure seeds of all the genotypes were collected from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. All the 10 rice genotypes were subjected to molecular characterization using Inter Simple Sequence Repeats (ISSRs) markers and the genotypes were grown in pots, in greenhouse, Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University. Seedlings of 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). The isolated DNA was purified using RNase and phenol, chlotroform and isoamyl alcohol precipitation. Concentration and quality of the isolated genomic DNA was determined by electrophoresis in an agarose gel and quantification was accomplished using spectrophotometer at 260 nm and 280 nm. The ratio of A260/A280 provided an estimate of purity of nucleic acid.

ISSR marker analysis

A total of 15 ISSR primers synthesized by Sigma Aldrich Chemical Pvt. Ltd. Bangalore, were used for PCR amplification. The details of ISSR primers used for PCR amplification are given in Table 1.

Table 1: Details of ISSR primers used for PCR amplification

SI. No.	ISSR-primers	Sequences (5'-3')
1.	UBC-807	AGAGAGAGAGAGAGAGT
2.	UBC-808	AGAGAGAGAGAGAGAGC
3.	UBC-809	AGAGAGAGAGAGAGAGG
4.	UBC-810	GAGAGAGAGAGAGAGAT
5.	UBC-811	GAGAGAGAGAGAGAGAC
6.	UBC-812	GAGAGAGAGAGAGAGAA
7.	UBC-820	GTGTGTGTGTGTGTGTC
8.	UBC-834	AGAGAGAGAGAGAGAGYT
9.	UBC-840	GAGAGAGAGAGAGAGAYT
10.	UBC-841	GAGAGAGAGAGAGAAYC
11.	UBC-844	CTCTCTCTCTCTCTCTRC
12.	UBC-853	TCTCTCTCTCTCTCTCRT
13.	UBC-857	ACACACACACACACACYG
14.	UBC-879	CTTCACTTCACTTCA
15.	UBC-880	GGAGAGGAGAGGAGA

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 deoxyribo 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 Tm requirement. The amplified products were separated in 3 per cent metamorpho agarose gel prepared in 1X TBE buffer stained with ethidium bromide (0.5 µl/ml). The gel was run in 1X TBE buffer (0.89 M Tris borate, 0.02 M EDTA, pH 8.0) at constant voltage of 80 V for a period of 2 hours to 2 hours 30 minutes. The gel was visualized in UV trans-illuminator and photographs were taken using gel documentation system (BIORAD Gel. Doc. XR imaging system).

Scoring and data analysis

All the genotypes were scored for the presence and absence of ISSR bands and the data were entered 1, 0 binary matrix for presence and absence of character for subsequent analysis using the computer package NTSYS-pc version 2.02. (Rohlf, 1998). PIC value of calculated for each of ISSR loci using the formula developed by (Rolden-Ruiz *et al.* 2000)

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

Where,

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

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).

Issr Analysis

The result obtained based on the analysis of 10 rice genotypes using 15 ISSR markers are furnished in Table 2. 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 bands 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 analyzed 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).

Table 2: Band variation and percentage of polymorphism in 10 rice genotypes

S. No	ISSR-primers	Total number bands	Number of monomorphic band	Number of polymorphic band	Percentage Polymorphism
1	UBC-807	40	7	33	82.5
2	UBC-808	20	2	18	90.00
3	UBC-809	41	4	37	90.24
4	UBC-810	23	7	16	69.56
5	UBC-811	12	6	6	50.00
6	UBC-812	54	10	44	81.48
7	UBC-820	5	5	0	0
8	UBC-834	26	8	18	69.23
9	UBC-840	6	4	2	33.33
10	UBC-841	22	8	14	63.63
11	UBC-853	2	2	0	0
12	UBC-857	13	10	3	23.07
13	UBC-879	28	10	18	64.28

Results and Discussion

In the present study, 15 ISSR primers were used to check polymorphism among the 10 genotypes of rice and 11 primers were found to be polymorphic (Fig. 1, 2 & 3). The 15 ISSR primers generated 292 markers for the assessment of genetic variability between the genotypes studied. The number of ISSR marker ranged from 3 as produced by the primer UBC-857 to 44 as yielded by primer UBC-820 among the 10 genotypes studied. Out of 292 ISSR markers amplified, only 209 markers were polymorphic which had resulted 71.5 per cent polymorphism. 11 primers produced polymorphic markers however the level of polymorphism percentage assorted with each primer ranged from 23.03 to 90.24 per cent. All the genotypes showed a varying degree of genetic diversity based on their amplification profile. The average percentage of polymorphism was 71.5 per cent. A high level of polymorphism was observed among the 10 genotypes studied. A similar and contradictory research findings were reported by Chaudhary *et al.* (2010), Hussain *et al.* (1989), Dongre *et al.* (2007) and Parkhiya *et al*. (2014). Chaudhary *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. A high level 71 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. The amplified ISSR fragments were in the range of 1700 bp to 5000 bp. The largest fragment of 5000 bp were amplified by the genotype ASD-20. Contradictory and similar findings were reported by Dongre *et al* (2007). Dongre *et al.* (2007) examined 19 ISSR primers, 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.

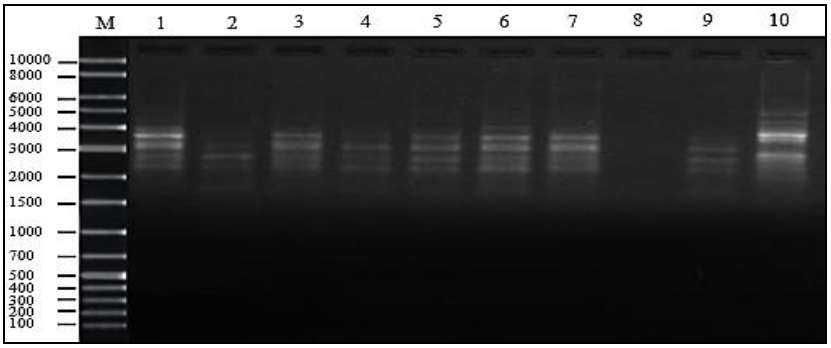

Fig 1: ISSR analysis with primer UBC-809 (AGAGAGAGAGAGAGAGG) on 10 genotypes of rice

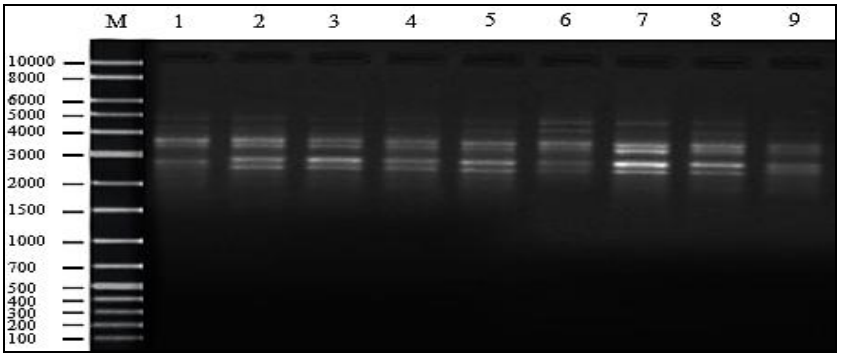

Fig 2: ISSR analysis with primer UBC-812 (GAGAGAGAGAGAGAGAA) on 10 genotypes of rice

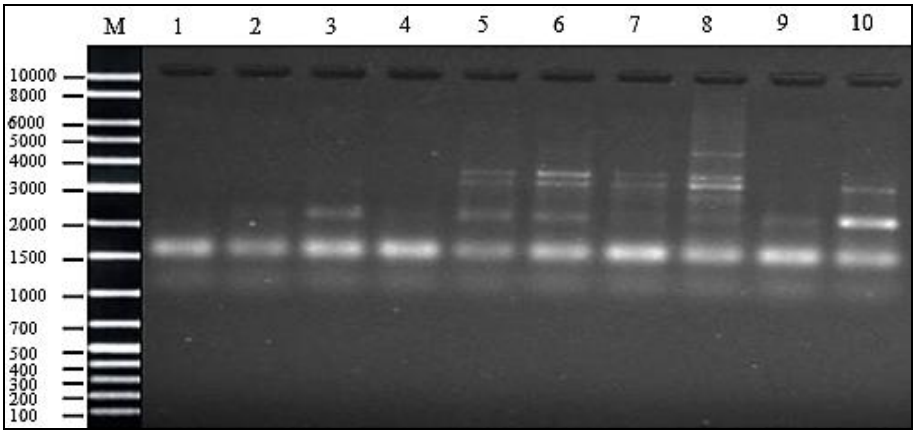

Fig 3: ISSR analysis with primer UBC-879 (CTT CAC TTC ACT TCA) on 10 genotypes of rice

Lane 1: ADT-36, Lane 2: ADT-37, Lane 3: ADT-38, Lane 4: ADT-39, Lane 5: ADT-42, Lane 6: CO-42, Lane 7: CO-43, Lane 8: CO-45, Lane 9: CO-47, Lane 10: ASD-20

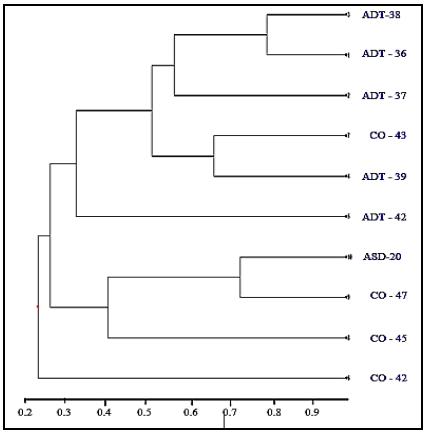

Fig 4: Dentro gram of 10 rice genotypes constructed from UPGMA cluster analysis using Jaccard's similarity coefficient based on data derived from 15 ISSR markers

Cluster analysis

Cluster analysis was erformed using the UPGMA method to group to the studied genotypes based on similarity coefficient. UPGMA was performed using Jaccard's similarity coefficient matrices calculated from 15 ISSR markers to generate a dendrogram for 10 rice genotypes. The coefficient value ranged from 0.25 to 1.0 indicated the genetic diversity among the 10 genotypes. The dendrogram showed the grouping pattern of three clusters. The 10 genotypes of rice were grouped into two main clusters (main cluster I and II) with an average of 40 per cent. The main cluster I consisted one genotype CO-42. The main cluster II considered of nine genotypes and it was divided into two sub clusters. (Sub clusters II A and II B). The sub cluster II A again divided in to two sub sub clusters as II Ai and II Aii. The sub sub cluster II Ai consisted of CO-45. The sub sub cluster II Aii consisted of two genotypes CO-47 and ASD-20. The sub cluster II B was divided into two sub sub cluster (II Bi and II Bii) the sub sub cluster II Bi consisted only one genotype ADT-42. The sub sub cluster II Bii consisted of five genotypes *viz.* ADT-39, CO-43, ADT-37, ADT-36 and ADT-38 shown in fig. 4.

Unique identification of rice genotypes

ISSR markers were used for the unique identification of 10 genotypes of rice. The unique markers were located across all the primers that individually identified each of the genotypes. Details of the genotypes specific marker generated by different primers are given in Table 3. The identification is based on presence and absence of unique marker. Eight genotypes could be identified on the basis presence of single unique marker. The ISSR primer UBC 808 could identify one genotype of rice ADT-42. The ISSR primer UBC-809 could identify two genotypes of rice (ADT-36 and ASD-20). The ISSR primer UBC-834 could identify two genotypes (ADT-38 and CO-47). UBC-840 could identify the genotype (ADT-39 and CO-450, UBC-841 could identify the genotype of ADT-36 and UBC-879 could identify the genotype CO-45.The ISSR primer UBC-834 and UBC-809 could identify the absence of marker in the genotype.

Table 3. Unique markers present/absent in rice geno types

Markers	Present/Absent	Genotypes identified
UBC-808	+	ADT-42
UBC-809	+	ADT-36,ASD-20
UBC-834	+	ADT-38,CO-47
UBC-840	+	ADT-39,CO-45
UBC-841	+	ADT-36
UBC-879	+	CO-45
UBC-809	-	CO-45
UBC-834	-	CO-42

Conclusion

The present investigation was undertaken with an objective of identifying distinguishable molecular markers for identification of 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 ten rice varieties using ISSR molecular markers. 15 ISSR primers were screened and 13 primers produced scorable bands. 11 ISSR primers showed polymorphism. A total of 292 ISSR markers were amplified, out of which 209 were polymorphic marker with 71.04 per cent polymorphism. The ISSR primers UBC-820 generated the maximum number of marker 44. The ISSR UBC-857 generated the least number of marker. The ISSR primer UBC-809 generated highly polymorphic profile. The ISSR primer UBC-857 produced low polymorphic profile. A total of 292 bands were obtained using 15 ISSR primer with an average of 22 alleles per primer 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 analyzed were 71.04 per cent. The Polymorphic Information Content (PIC) for the primer ranged from 0.0994 (UBC-820) to 0.8750 (UBC-841). The 10 genotypes of rice were grouped into three main clusters (main clusters I, II and III) with an average of 40 per cent. The main cluster I

consisted two genotypes it was divided into two sub clusters (sub clusters IA and IB). The sub cluster IA consisted of two genotypes CO-43 and ADT-39. The ISSR primer UBC-834 could identify two genotypes *viz.* ADT-38 and CO-47. UBC-840 could identify the genotypes ADT-3, CO-45 and UBC-841 could identify the genotypes ADT-36 and UBC-879 could identify the genotype CO-45. The ISSR primer UBC-834 and UBC-809 could identify the genotypes CO-42 and CO-45.

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