Genetic Diversity Assessment In Deep Water Rice Found In Manipur, India Using Molecular Markers

Chongtham Henary Singh, Van Cuu Nguyen, Vanga Sanga Reddy, Sadhu Leelavathi, Guruaribam Shantibala Devi

Abstract: Molecular markers were used to study genetic diversity among the eight selected rice genotypes where six are local genotypes found in Manipur. A total of 19 RAPD, 17 ISSR and 29 SSR primers were screened where 13 RAPD, 12 ISSR and 20 SSR generated a total of 90, 88 and 160 alleles were detected respectively. The average number of alleles per locus was 6.9, 7.3 and 8.0 for each marker. The polymorphic information content (PIC) value ranged from 0.38 to 0.86 in RAPD, 0.16 to 0.79 in ISSR and 0.54 to 0.88 with SSR analysis. A combined cluster analysis was performed using UPGMA using the DICE's similarity coefficient and the resulting dendrogram resolved the selected rice cultivars into two major clusters value ranging from 0.66 to 0.79. From the results, it is proven that this study can be useful in polymorphism analysis and genetic improvements by breeding in future.

Index Terms: Genetic diversity, RAPD, ISSR, SSR, DICE, Dendrogram.

1 INTRODUCTION

Rice (*Oryza sativa* L.) is the most important food crops which is widely consumed as staple food over half the world's population especially in Asia. It accounts for more than one-third of global population and also supply over half of the world rice production [4]. In addition to the two major subspecies, japonica and indica, [15] several other minor rice types have been identified with genetic markers. As a consequence of adaptations to different habitats, extensive genotypic and phenotypic diversity exists within O. sativa, resulting in about 120,000 different accessions [12].

Molecular markers technique have been proven powerful in estimation understanding genetic variations in plant genomes. Therefore, DNA finger printing for cultivar or varietal identification has become an important parameter for genetic signature in plant breeding and germplasm management. It includes PCR based multiple loci techniques such as RAPD (Randomly Amplified Length Polymorphism), AFLP (Amplified fragment length polymorphism), ISSR (Inter simple sequence repeat) and SSR (Simple sequence repeats) or micro satellites [23] are playing important role in crop improvement [7]. These techniques help to take fingerprints of a species in revealing polymorphism at molecular level which could be caused due to differences of a single-nucleotide sequence at the priming sites such as point mutations or by structural arrangements within the amplified sequence e.g., insertions, deletions, inversions.

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SSR are considered to be the most suitable markers due to their multiallelic nature, high reproducibility, codominant inheritance, abundance and deals with point mutations, extensive genome coverage [13]. RAPD deletions and insertions which polymorphism to be detected [32]. In influence the base sequence of primer binding sites, allowing addition, it also shows higher level of reproducibility and cost-effectiveness per polymorphism. ISSR uses microsatellite, usually 16-25 bp long as primers in a single primer reaction targeting multiple genomic loci to amplify mainly the inter SSR sequences of different sizes [35] and fingerprinting of varieties [33]. Various molecular marker techniques such as RFLP- Restriction fragment length polymorphism [34], RAPD [30], AFLP [8], ISSR [11] and [5] and SSR [3] have been used for analysis of rice germplasm in different regions. The Northeastern region of India has been identified as biodiversity 'hotspot', it is rich in both flora and fauna diversities [18]. In this region, different rice varieties are cultivated under several conditions such as upland, lowland or deep water. In Manipur, local farmers are cultivating many indigenous rice varieties which may contain a considerable genetic diversity that can serve as a source of germplasm for genetic improvements of cultivated varieties of rice. The use of molecular markers have been benefited in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species of rice. The present study was undertaken for the assessment of genetic diversity among the selected rice cultivars with the help of RAPD, ISSR and SSR markers.

2 MATERIALS AND METHODS

2.1 Plant materials

Seeds of six genotypes of rice: Taothabi (TT), Laiphou (LP), Khongan (KG), Moirangphou khongangbi (MP), Kumbiphou (KP) and Sung sungba (SS) from different location of Manipur and two varieties: IR64 (IR) and Nipponbare (NB) from IARI, Pusa, New Delhi were collected. The selected eight were used to study for analyzing genetic diversity.

2.2 Isolation of Genomic DNA

Healthy seeds of each sample were sowed in plastic cups containing soil and water for germination. Genomic DNA isolation was carried out following the protocol of [16] with minor modifications. The quality and quantity of DNA was assessed by NanoDrop 1000 spectrophotometer.

2.3 PCR preparation

PCRs were carried out in 10 µl of reaction mixture containing 5–50 ng of template DNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 2 mM MgCl2, and 0.5 U of Dream Taq DNA Polymerase (Fermentas, Canada).

2.3.1 RAPD analysis

19 decamer custom primers (IDT, USA) were used: Amplification was carried out under the following conditions: 95 °C for 5 min, 40 cycles of 94 °C for 30 sec, 37–39 °C for 1 min, and 72 °C for 1 min, and then 72 °C for 7 min. The amplification products were checked on a 1.5 % agarose gel.

2.3.2 ISSR analysis

17 primers (IDT, USA) were used: Amplification was carried out under the following conditions: 95 °C for 7 min, 40 cycles of 95°C for 1 min, 43-52 °C for 1 min, and 72 °C for 2 min, and then 72 °C for 7 min. The amplification products were checked on a 1.5 % agarose gel.

2.3.3 SSR analysis

29 standard SSR primers (IDT, USA) were used: Amplification was carried out under the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 1min, 55–60 °C for 1 min, and 72 °C for 2 min, and then 72 °C for 7 min. The amplification products were checked on a 8 % polyacrylamide gel.

2.4 Data analysis

The amplified bands for each markers were scored using a binary scoring system that recorded the presence and absence of bands as "1" and "0" respectively. The resultant matrix was used to calculate genetic similarities based on Dice coefficient [14] were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. Cluster analysis were carried out in NTSYS (Numerical Taxonomy and Multivariate Analysis System) version 2.2. A dendrogram was constructed by using UPGMA (Unweighted Pair Group Method with Arithmetic mean) [21] to group individual into different clusters.

PIC values were calculated as described by [22] with the following formula:

$PIC_i = 1 - \sum_{i=0}^{n} P_{ij}^2$ where Pij is the frequency of the jth allele for the ith marker.

3. Results and discussion

3.1 RAPD analysis

For analyzing the diversity among the selected 8 rice genotypes, 19 RAPD primers were used from which 13 primers generated polymorphic bands (Figure 1A) and a total of 90 alleles were detected. The number of alleles per locus varied from 2 to 10. The average number of alleles per locus was 6.9. The overall size of amplified products ranged from 250b (OPA-03) to 3000 bp (OPB-18). The percentage of polymorphism obtained by these 13 primers was similar when compared to RAPD analysis in rice genotypes by [25] with 81.2% of polymorphic products. The primer OPB08 gave the minimum number of fragments (2), while the highest number of fragments (10) were amplified with four primers OPA03, OPA08, OPA18 and OPB18. The PIC value for the RAPD ranged from 0.38 to 0.86 with an average of 0.63 (Table 1) and the result was similar with that reported by [26] and [27]. The highest PIC value (0.88) was observed for primer OPB-18. Cluster analysis was calculated based on Dice's similarity coefficients matrices from RAPD markers. This analysis generate a dendrogram for the selected genotypes which revealed two major clusters at a similarity coefficient of 0.59 (Figure 1B). Cluster I was the largest and included six local genotypes while cluster II consisted of two known genotypes i.e. NB and IR. With Dice coefficient of similarity revealed that 0.80 similarity exist between genotypes MP and SS whereas similarity between TT and IR was found to be very low with coefficient of 0.59. The major cluster I further divided into two sub clusters IA and IB. The sub cluster IA consist of two genotypes which are with the similarity coefficient at 0.79 between TT and LP. While sub cluster IB consist of four local genotypes namely KG, KP, MP and SS with similarity coefficient from 0.78 to 0.80. This study indicates that RAPD is an effective and powerful technique in detecting genetic variation among rice cultivars. [1] stated that RAPD analysis is a simple and sensitive technique which can be used to detect polymorphism in rice at DNA level.

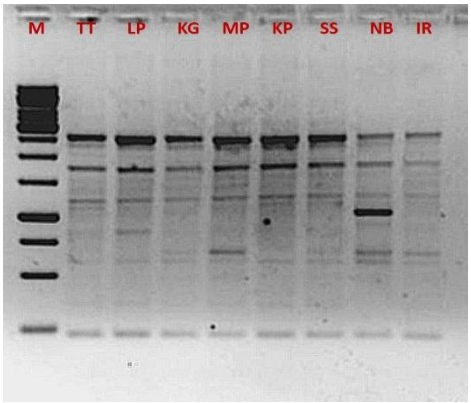

Figure 1A. *RAPD profile using primer OPA03 of eight genotypes of rice where M- 1kb DNA ladder*

Table 1. *Details of RAPD primers used in studies*

Sl. No.	Primers	Sequence 5'-3'	Total no. of bands	No. of Polymorphic bands	No. of Monomorphic bands	PIC values
1.	OPA-01	CAGGCCCTTC	9	8	1	0.70
2.	OPA-02	TGCCGAGCTG	5	4	1	0.70
3.	OPA-03	AGTCAGCCAC	10	7	3	0.78
4.	OPA-04	AATCGGGCTG	0	0	0	0.00
5.	OPA-08	GTGACGTAGG	10	9	1	0.70
6.	OPA-09	GGGTAACGCC	6	5	1	0.44
7.	OPA-10	GTGATCGCAG	0	0	0	0.00
8.	OPA-11	CAATCGCCGT	8	8	0	0.62
9.	OPA-13	CAGCACCCAC	0	0	0	0.00
10.	OPA-14	TCTGTGCTGG	3	2	1	0.59
11.	OPA-16	AGCCAGCGAA	4	3	1	0.59
12.	OPA-17	GACCGCTTGT	0	0	0	0.00
13.	OPA-18	AGGTGACCGT	10	10	0	0.77
14.	OPB-07	GGTGACGCAG	9	9	0	0.44
15.	OPB-08	GTCCACACGG	2	1	1	0.38
16.	OPB-12	CCTTGACGCA	0	0	0	0.00
17.	OPB-13	TTCCCCCGCT	4	2	2	0.70
18.	OPB-17	AGGGAACGAG	0	0	0	0.00
19.	OPB-18	CCACAGCAGT	10	7	3	0.86
		Total	90	75	15	8.27
		Average	6.9	5.77		0.63

3.2 ISSR analysis

17 ISSR primers were used for analyzing genetic diversity and relationship, 12 primers generated 75 polymorphic and 15 monomorphic bands among the genotypes (Figure 2A) and a total of 88 alleles were detected among the selected 8 rice genotypes. The number of alleles per locus varied from 4 to 10. The average number of alleles per locus was 7.3. The overall size of amplified products ranged from 300 (U820) to 3000 bp (U841). The primer U864 gave the minimum number of fragments (4), while the highest number of fragments (10) were amplified with three primers U841, U836 and U820. The average PIC value was 0.54 and the highest and lowest PIC values were 0.79 and 0.16 respectively (Table 2). The highest PIC value (0.79) was observed for primer U820. [24] reported

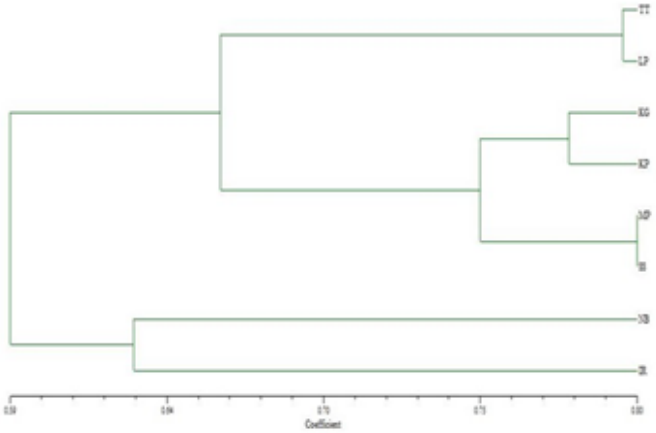

Figure 1B. *A dendrogram showing clustering of the eight genotypes of rice based on RAPD data*

that similar lowest PIC value during his investigation of rice varieties. While the highest and the average PIC values was found to be as that of [28]. Cluster analysis was calculated based on Dice's similarity coefficients matrices from ISSR markers. This analysis generate a dendrogram for the selected genotypes which revealed two major clusters at a similarity coefficient of 0.61 (Figure 2B). NB, a japonica variety was the most genetically dissimilar and was segregated from all other genotypes. Cluster I consist of one local and one wild genotype while cluster II consisted of five (one known and four local) genotypes. In cluster I, TT and NB was found to have similarity coefficient of 0.74 whereas in cluster II, it can be further divided into two sub clusters IA and IB in which clusters IA, KG, and KP show similarity coefficient of 0.74 while in cluster IB, it consist of three genotypes i.e. MP, SS and IR with similarity coefficient from 0.70 to 0.77.

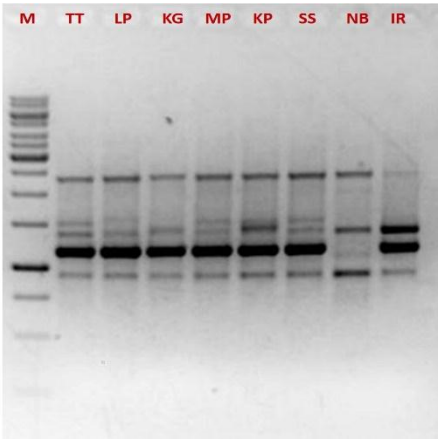

Figure 2A*. ISSR profile using primer U807 of eight genotypes of rice where M- 1kb DNA ladder*

This study proved that the selected ISSR primers markers can detect polymorphism within and among populations and/or species of rice. [29] and [31] reported that ISSR is a powerful

marker for analyzing the genetic and its phylogenetic relations fingerprinting in the genus *Oryza*.

Table 2. *Details of ISSR primers used in studies*

Sl. No.	Primers	Sequence 5'-3'	Total no. of bands	No. of Polymorphic bands	No. of Monomorphic bands	PIC values
1.	UBC-807	(AG)7T	8	5	3	0.77
2.	UBC-808	(AG)7AC	7	7	0	0.77
3.	UBC-809	(AG)8G	6	4	2	0.60
4.	UBC-811	G(AG)7AC	6	5	1	0.49
5.	UBC-813	(CT)8T	6	5	1	0.70
6.	UBC-814	(CT)8A	0	0	0	0
7.	UBC-817	(CA)8A	8	7	1	0.70
8.	UBC-818	(CA)8G	0	0	0	0
9.	UBC-820	(GT)8C	10	10	0	0.79
10.	UBC-825	(AC)8T	6	4	2	0.72
11.	UBC-827	(AC)8G	0	0	0	0
12.	UBC-830	(TG)8G	7	6	1	0.66
13.	UBC-836	(AG)8YA	10	9	1	0.67
14.	UBC-841	(GA)8YC	10	8	2	0.74
15.	UBC-847	(CA)8RC	0	0	0	0
16.	UBC-848	(CA)8RG	0	0	0	0
17	UBC-864	(ATG)6	4	4	0	0.16
		Total	88	74	14	7.07
		Average	7.3	6.16		0.54

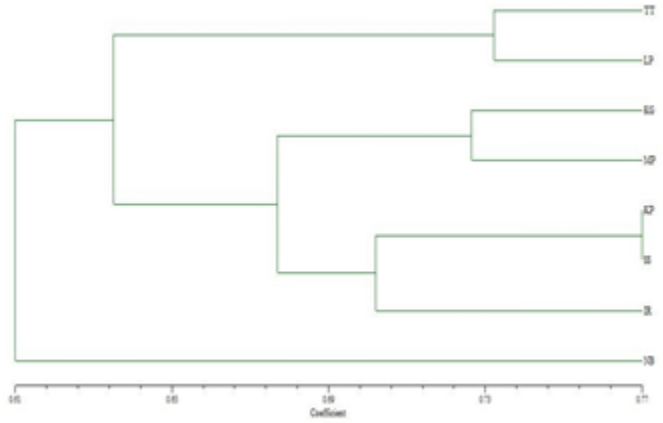

Figure 2B. *A dendrogram showing clustering of the eight genotypes of rice based on ISSR data*

3.3 SSR analysis

29 SSR primers were used for testing the diversity and a total of 160 alleles were detected among the selected 8 rice genotypes. The number of alleles per locus varied from 5 to 10. The average number of alleles per locus was 8. The overall size of amplified products ranged from 100 (RM105) to 3000 bp (RM316). Among the 26 ISSR primers used for analysis of genetic diversity and relationship, 20 primers generated 121 polymorphic and 39 monomorphic bands among the genotypes (Figure 3A). The primer RM283 gave the minimum number of fragments (5), while the highest number of fragments (10) were amplified with five primers RM5, RM161, RM125, RM307, RM536 and RM352. The PIC value for the SSR ranged from 0.54 to 0.88 with an average of 0.54 (Table 3). Different ranges of PIC values have been reported on rice varieties of different ecotypes by using SSR markers namely 0.4039 to 0.5840 with an average of 0.4872 [9], 0.10 to 0.50 with an average of 0.31 [33], 0.47 to 0.88 with an average of 0.71 [17] and 0.059 to 0.929 with an average of 0.665 [19]. The highest PIC value (0.88) were observed for primers RM307 and RM536. [20] and [10] stated in their study that PIC values determined the level of genetic diversity and depend upon factors such as breeding behavior of the species, genetic diversity in the collection, size of the collection, sensitivity of genotyping method and location of primers in the genome used for study. The cluster analysis showed a significant genetic variation among the genotypes with DICE similarity coefficients ranging from 0.70 to 0.80 (Figure 3B). The dendrogram revealed two distinct clusters at a similarity coefficient level of 0.70. Cluster II was the largest and included five genotypes while, clusters I comprised three genotype. The cluster II further divided into two sub clusters IIA and IIB. II A consisted of two genotypes while II B consisted of three genotypes. TT and IR were distantly related at a similarity coefficient of 0.70. Cluster analysis showed that TT and LP showed maximum similarity with the similarity coefficient of 0.80. Based on dendrogram, the genotypes TT and LP were closely related to NB while the remaining local cultivars were in same cluster with IR. All the six local cultivars were found to be genetically diverse. [12] reported that for maintaining future food security, SSR markers proved to be crucial for rice breeding programs, especially for genotyping, diversity analysis and genetic mapping for vast genetic diversity found in indigenous rice varieties in NE India. [27] also highlighted that SSR markers can be used to study regional genetic diversification of rice found in North Eastern India.

Table 3. *Details of SSR primers used in studies*

Sl. No.	Primers	Total no. of bands	No. of Polymorphic bands	No. of Monomorphic bands	PIC values
1.	RM5	10	6	4	0.85
2.	RM11	0	0	0	0
3.	RM19	7	6	1	0.81
4	RM25	0	0	0	0
5.	RM105	9	6	3	0.86
6.	RM124	0	0	0	0
7.	RM125	10	7	3	0.86

8.	RM133	9	8	1	0.65
9.	RM154	7	5	2	0.78
10.	RM161	10	9	1	0.73
11.	RM162	0	0	0	0
12.	RM237	6	4	2	0.73
13.	RM259	6	6	0	0.62
14.	RM271	7	6	1	0.59
15.	RM277	0	0	0	0
16.	RM283	5	3	2	0.77
17.	RM287	0	0	0	0
18.	RM307	10	8	2	0.88
19.	RM316	7	4	3	0.72
20.	RM338	9	5	4	0.86
21.	RM408	0	0	0	0
22.	RM413	9	9	0	0.54
23.	RM452	0	0	0	0
24.	RM484	0	0	0	0
25.	RM495	5	4	1	0.70
26.	RM507	7	4	3	0.84
27.	RM514	7	4	3	0.79
28.	RM536	10	8	2	0.88
29.	RM552	10	8	2	0.85
Total		160	121	39	15.31
Average		8	6.05		0.54

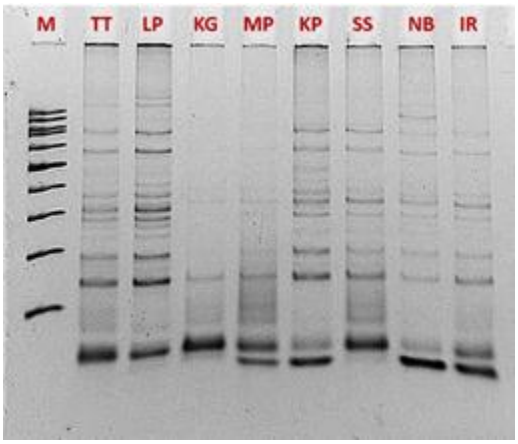

Figure 3A. *SSR profile using primer RM536 of eight genotypes of rice where M- 100bp DNA ladder*

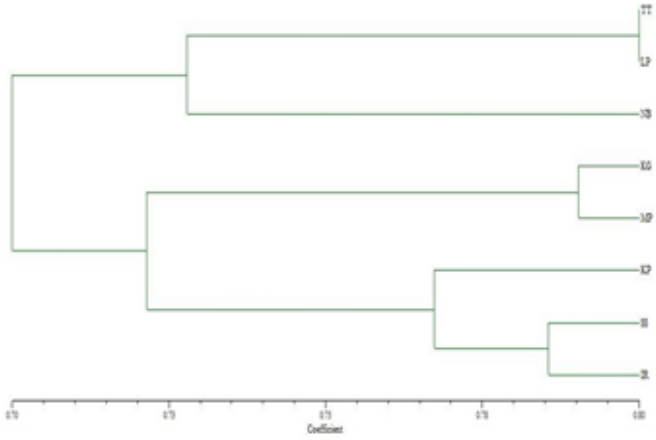

Figure 3B. *A dendrogram showing clustering of the eight genotypes of rice based on SSR data*

3.4 Combined analysis of RAPD, ISSR and SSR

The combined cluster analysis of three markers generated a dendrogram with two major clusters in which NB stands alone from the two clusters indicating its genetic difference from the remaining genotypes. This dendogram revealed that TT and LP showed the highest similarity followed by SS. Cluster II was the largest and consist of five genotypes, namely KG, MP, KP, SS and IR with the similarity coefficient that ranged between 0.73 to 0.78 while clusters I comprised two genotypes namely TT and LP, with the similarity coefficient at 0.79 (Figure 4). In cluster II, it has two sub cluster IIA and IIB, where IIA contained KG and MP with a similarity coefficient at 0.77 while IIB contained two sub-clusters where the first consist KP and the second consist of SS and IR. Thus, this analysis proved to be significant in representing the genetic dissimilarities among the eight genotypes.

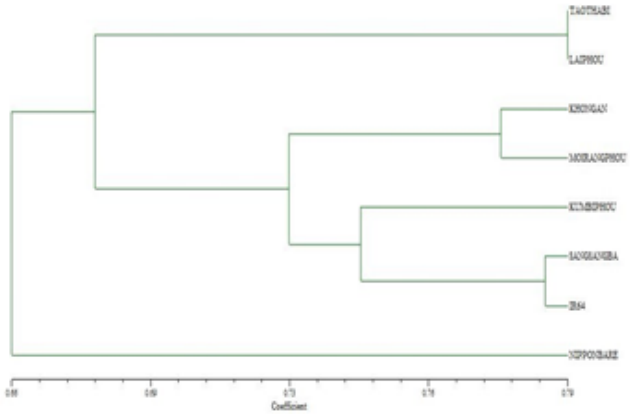

Figure 4. *A dendrogram showing clustering of the eight genotypes of rice based on combined datas of RAPD, ISSR and SSR*

4. Conclusion

In the present study, among the three markers, SSR showed the highest PIC value followed by RAPD while ISSR showed the lowest PIC value. All the markers proved to be very

efficient in characterizing the genetic polymorphism within the selected rice genotypes. This is the first report for studying genetic relationships using molecular markers in deep-water rice of Manipur with known varieties. This study revealed that these markers can play a key role for analysis of genetic diversity, germplasm conservation, identification and selection of suitable rice germplasm in future for genetic improvements.

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