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Assessment of genetic diversity of rice germplasm using microsatellite markers

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Abstract
The knowledge on the extent and pattern of diversity in the crop species is a prerequisite for any crop improvement program as it helps breeders in deciding suitable breeding strategies for their future improvement. The study was conducted to analyze the genetic diversity among 77 germplasm lines at molecular level employing 36 randomly chosen microsatellite markers. A total of 30 markers were found to be polymorphic among the genotypes with a total of 70 alleles. The number of alleles per locus ranged from 2 to 3. The polymorphic information content (PIC) value ranged from 0.072 to 0.640 with an average of 0.32. Based on the principle of Unweighted Pair Wise Method using Arithemetic Average (UPGMA) constructed by Jaccard's similarity Coefficient, the cluster analysis distinguished these accessions into eight clusters. Cluster I had maximum number of genotypes (59) followed by cluster II (8), clusters III (3), whereas the clusters IV and VII have two genotypes each and cluster V, VI and VIII had only one genotype. Thus, the germplasm lines present in the eight clusters were genetically diverse and could be directly utilized in hybridization programme for improvement of yield and yield related traits.

Keywords: Rice, genetic diversity, SSR markers, PIC value, dendrogram

Introduction
Rice (*Oryza sativa* L.) belonging to the family Gramineae and is also called as the 'Grain of Life' (Fiyaz *et al*., 2011) [4], it is one of the most important food crop for about half of the population in the world (Rao *et al*., 2019) [11]. In India, rice crop occupies a pivotal role with largest area under cultivation and has maximum share in production and productivity. The global consumption rate and the total demand for rice continues to rise (Khush, 2005) [8]. To meet the demand of ever increasing population for rice, there is an urgent need to exploit the genetic diversity and variability in plant breeding programmes. Among all the centres for rice diversity, India is one of the largest center of diversity and the accessions of rice are a rich reservoir of useful genes with wide genetic variability, leaving a wide scope for the rice improvement programme (Singh *et al*., 2015) [17].

Genetic diversity of germplasm occupy an important position in breeding and crop improvement program as it helps in analyzing and establishing genetic relationship in accessions collection, monitoring and identification of diverse parental combinations to create segregating progenies with high genetic variability (Ramadan *et al*., 2015; Islam *et al*., 2012) [13, 6]. Genetic diversity can be determined by assessment of morphological and or molecular data. Morphological/phenotypic expression of a plant is affected by the environment; therefore, selection based on morphological traits is seductive and it also has disadvantages in terms of time, space, and labor cost. Unlike morphological traits, molecular markers have become quite handy in precisely understanding the extent of genetic divergence among varieties. Evaluation of genetic diversity using molecular marker technology is non-destructive, requires small number of samples, not affected by environmental factors, and does not require large experimental setup and equipment for measuring physiological parameters (Kanawapee *et al*., 2011) [7].

Among all the available molecular markers, Simple sequence repeat (SSR) is one of the best marker technology for identification of genetic diversity of accessions (Sajib *et al*., 2012) [16]. SSR markers are codominant, highly polymorphic, able to detect high level of allelic diversity, informative, easily analyzed and cost effective (Garcia *et al*., 2004) [5] thus being widely applied in genetic diversity analysis, gene mapping and molecular map construction

(Zhang *et al*., 2007) [20]. Due to their multi-allelic and highly polymorphic nature, even less number of SSR markers can give a better genetic diversity spectrum (Singh *et al*., 2016) [18]. Hence, the present study was undertaken with the aim to assess the genetic diversity in seventy-seven germplasm lines of rice using SSR markers. The information generated from this study will enable better selection of diverse parents and to select appropriate parental genotypes in breeding programme.

Materials and Methods

The experimental material consisted of 77 germplasm lines. The field experiments were conducted at ICAR-Indian Institute of Rice Research (ICAR-IIRR), Rajendranagar, Hyderabad, India during *kharif* 2019 and 2020. Seeds were sown in nursery on raised beds and thirty days old seedlings of each genotype were transplanted in two rows with 20 plants per row following a spacing of 20 cm between rows and 15 cm between plants. The experiment was laid in Randomized Block Design (RBD) with two replications. Recommended agronomic and plant protection measures were taken up for raising a healthy nursery and main crop.

DNA extraction and PCR amplification

Healthy leaf samples of 2-3 cm long were collected from young plants and DNA was extracted using Cetyl trimethyl ammonium bromide (CTAB) protocol as per Doyle (1991). DNA concentration was estimated using agarose gel electrophoresis and DNA samples were diluted with 1x TE into an equal concentration of 25 ng/µl.

Amplification of the markers using polymerase chain reaction (PCR) was done with a 10 µl reaction volume with 3 µl of 25 ng DNA template, 1 µl of 10x PCR buffer and MgCl, 0.3 µl of each forward and reverse primers, 1 µl of 1 mM dNTP, 4.5 µl sterile distilled water and 0.2 µl of 5 U of *Taq* polymerase (Bangalore Genei, India). The following PCR profile was used for amplification in the thermocycler (Eppendorf, USA): initial denaturation at 94º C for 5 min and then 35 cycles of denaturation at 94º C for 45 s, annealing at 55º C for 45s and extension at 72º C for 1s; final extension at 72º C for 10 min and cooling at 4º C. Finally, 3 µl of 1x loading buffer for every 10 µl of PCR product was added to the PCR product prior to loading. The PCR products were resolved by electrophoresis using a 3.5% agarose gel electrophoresis (Thermofisher Scientific, USA). The gel was run in 1x TAE at 120 V for 2 hours depending on the product size of the marker and were visualized under the UV-transilluminator (Biorad) and documented and stored using GELSTAN.

Data analysis

Genetic diversity was estimated using 30 polymorphic SSR markers (Table 1). Polymorphic Information Content (PIC) was calculated as described by Botstein *et al*. (1980)[2] using the below formula;

$$PIC = 1 - \left[\sum_{i=1}^{n} Pi^2\right] - \left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2Pi^2Pj^2\right]$$

Where p equals the frequency of the i^{th} allele and p the frequency of the (I +1) th allele. For diversity analysis, only data from polymorphic loci was used. The binary data matrix generated by polymorphic SSR markers were subjected to further analysis using NTSYS-pc version 2.11W (Rohlf, 1997) [15]. The SIMQUAL program was used to calculate the Jackard's dissimilarity coefficient. The dissimilarity matrix was used as an input for analysis of clusters. UPGMA-based clustering was done using SAHN module of NTSYSpc for dendrogram construction. Based on Unweighted pair-group average (UPGMA) method, the clusters are joined based on the average distance between all members in the two groups.

Results and Discussion

Number of alleles and PIC value

In the present study, considerable amount of genetic variability was found among 77 germplasm lines. The level of polymorphism among the germplasm lines was evaluated by calculating the number of alleles and PIC values using PowerMarker software. A total of 36 SSR randomly chosen markers that covered 12 chromosomes, were used to assess the extent of genetic diversity across 77 rice genotypes. A total of thirty markers produced reproducible polymorphic pattern while remaining six primers were monomorphic. These 30 markers showed a total of 70 alleles and the number of alleles per locus ranged from 2 to 3. Among the polymorphic markers, 20 markers produced 2 alleles each and 10 markers produced 3 alleles. The overall size of amplified products varied from 50 (RM 156) to 477 bp (RM 206). The polymorphic information content (PIC) is the reflection of allele diversity and their frequency among genotypes. PIC values ranged from a 0.0721 to 0.6400 with an average of 0.32 (Table 1). The marker RM22586 showed highest PIC value and lowest PIC value was obtained for RM19665.

Diversity analysis

The Jaccard's similarity coefficient varied from 0.44-0.93 as revealed by UPGMA cluster analysis using thirty markers. The germplasm lines close to the similarity coefficient of 0.44 were considered as more dissimilar, while the germplasm lines close to the similarity coefficient of 0.93 as similar. On the basis of dendrogram, the highest similarity coefficient value was observed between the cultivar BPT 5204 and Jaya (0.93) followed by CR Dhan 500 and Tetep (0.91), whereas lowest value was observed for Pusa Basmati 1121 (0.44) followed by Improved Samba Mahsuri and ADT 49 (0.47). Thus, these accessions were genetically diverse and could be directly utilized in hybridization programme for improvement of yield and related traits.

A dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA method. The seventy-seven germplasm lines were grouped into eight main clusters (Fig. 1) at a cut-off similarity coefficient of 0.524. The germplasm lines which are of genetically similar are grouped in one cluster. Each cluster distinguishes the genotypes clearly from the other. In the dendrogram, cluster I had maximum fifty-nine genotypes followed by cluster II had eight genotypes, clusters III have three genotypes each, whereas the clusters IV and VII have two genotypes each and cluster V, VI and VIII had only one genotype each. Cluster I was the major cluster with 51 genotypes and divided into three sub clusters IA, IB and IC. The sub cluster IA with 31 genotypes, was divided further into two sub clusters IA-1 and IA-2 at similarity coefficient (0.57), the sub cluster IA-1 has 29 genotypes and sub cluster IA-2 had two genotypes *viz*., Sabita and CSR 10. Sub cluster IB had 24 genotypes and Sub cluster IC had three genotypes *viz*., Dinesh, Pushyami and CSR 36. Cluster II was

divided into two sub clusters IIA and IIB at similarity coefficient (0.584). Sub cluster IIA have six genotypes and IIB has two genotypes WGL 14 and CR Dhan 202 at similarity coefficient (0.7). Cluster III has three genotypes *viz*., PR 124, MAS 9461 and Kalanamak. Cluster IV has two genotypes *viz*., PB 1 and RNR 15048 at similarity coefficient (0.56), whereas cluster V and VI had one genotype each, Vivek Dhan 62 and Badshabhog at similarity coefficient (0.517, 0.47). Cluster VII had two genotypes (ADT 49 and ISM) with similarity coefficient 0.63 and cluster VIII had only one genotype PB 1121 with low similarity coefficient of 0.44. Clustering pattern is presented in the Table 2.

The germplasm lines under evaluation in the present investigation are fixed lines with high yielding and quality obtained from all over the India. Further characterization of such germplasm lines with markers was done to ascertain their diversity and applicability for selection of desirable parents in breeding programmes. Number of alleles per marker obtained in the present study are comparable to the earlier findings by Rachana *et al*. (2019) [12] and Rashmi *et al*. (2017) [14]. The PIC value observed in the present study are also comparable to the earlier reports by Beser and Mutafcilar, (2021) [1], Kumar *et al*. (2021) [9] and Verma *et al*. (2019) [19]. The higher the PIC value of a locus, the higher the number of alleles detected. The markers RM206, RM495, RM11033, RM10162, RM15404, RM19609, RM22586, RM25066, JGT 8-10.2, JGT 11-15.3 and RM24954 were found to be the most appropriate marker to discriminate among the rice germplasm lines used in the present study owing to the highest PIC value of 0.5-0.64.

Jaccard's similarity coefficient describes the relatedness among the genotypes. The higher the genetic difference among the genotypes, more clusters are obtained with single genotype in each cluster (Nihar *et al*., 2016) [10]. In the present study we observed three single genotype clusters in the dendrogram. Among all the genotypes, Pusa Basmati 1121 singled out with least similarity coefficient of 0.44 as an independent cluster. It is known for its extraordinary kernel (grain) length of 8.4 millimetres, the longest-ever known released cultivar in the world. It has very high kernel elongation ratio ranging from 2 to 2.5. When cooked, the rice does not turn sticky, possesses minimum breadth-wise expansion, and is aromatic with intermediate alkali-spreading value and amylose content. Hence, the marker based information supports the morphological diversity present in them. Similar results were reported by Farhzadi *et al*., (2020) [3] in 30 cultivars of Indica rice using SSR markers and the genotypes were grouped in to three major clusters. Rachana *et al*. (2019) [12] reported the grouping of fourty six core set of rice accessions using 67 SSR markers in to eight clusters. Similarly, Beser and Mutafcilar (2020) [1] reported the grouping of the 60 rice varieties into two main clusters using 50 markers distributed across all the chromosomes. The microsatellite marker alleles provided a greater degree of discrimination between the accessions than the quantitative characterization. The more allelic diversity among the genotypes clearly emphasizes on the scope for improvement of yield and related genes by introgression of genes from genotypes of different clusters and could be directly utilized in hybridization programme.

Table 1: List of markers used in the present study for genetic diversity

Sl. No	Marker	Chr.	Forward Sequence	Reverse Sequence	No. of alleles	PIC
1	RM 495	1	ATGATGATGGACGACGACAACG	TGAATCCAAGGTGCAGAGATGG	2	0.6075
2	RM 11033	1	TGTTTCAGAGTTCAGAGTCACACG	AGGACAAGCCCACTTATTGAACC	3	0.6310
3	RM 10162	1	GGGCATGTCATTTCCTACTCCTACCG	CCACAAAGTCGCAATGTCCTGTAGC	3	0.5107
4	RM 13070	2	CCACCGGAGATCATAGGTTCG	AAGTTAGCCCTCAACCGCTATGG	2	0.2730
5	RM 15404	3	TTTGGCAAGGCTATCTTCTCTGG	GGAGAGCCGAAACTATTTGATTGG	3	0.6174
6	RM 489	3	GAACAGGGACACAATGATGAGG	GACGATCGGACACCTAATTACAGC	2	0.1864
7	RM 16791	4	GGCTGCTTCAGAGCGTTTGTAGG	AAATACGCCATCAAGTTGCACACC	2	0.3392
8	RM 16649	4	CTCCCTTCATGCGTAAGCTCTCC	GCAAACAGGATCCTCCACAAAGG	2	0.3215
9	RM 1386	5	TCAAGCTGCATTAGGAAGACACC	AACTTAGCTGAAACGCAACACG	2	0.3156
10	RM 18516	5	CTTTCGTCCTGTACGTAAACC	TCAAACTACCCTCACATTCTCC	3	0.1668
11	RM 17950	5	GGAAATGTGCATAGGTAGTTCAGG	GAGTTGGGAACTGCTACAAACG	2	0.2349
12	RM 19609	6	GTTTGTGGACATCGTTGTTGTGC	CGGATTGCAGCCTTAGTTGTACC	2	0.5347
13	RM 3567	6	ACCACGCGTCATTGACATCC	ATGGGATGAAACTGCCACAACC	2	0.2349
14	RM 20098	6	GGATACGCGATACGAGGATACG	TCAGCGTGAATTAGCTACAAGAGG	3	0.3465
15	RM 22586	7	TCACCAACAAGTGGAGTACTTAGGC	CCTTACCTCAGGAGTGTTCGATCC	3	0.6400
16	RM 21539	7	GCCCAACTACTTCGACAGCTTCC	CAATGACCTGAGTAGCATCCAAGG	2	0.4025
17	RM 21309	7	GAGTGGATTTACTGACAAGCTCTTCC	GATTCTCTTGGAGCCCATACTCC	3	0.5441
18	JGT 8-10.2	8	GTACTAAACAGATGGAGCAGTACG	GTGGTACTGCTAATTAGGGGGTA	2	0.5533
19	RM 22839	8	ACGGTACGGATTATAGCGATGC	ACGCTCATCAACCGAGAAAGG	2	0.0750
20	RM 22772	8	GCCCGAAGAAATCAGAGTAGTAAGG	TTAGCAATATGGTGCCCAAGTCC	2	0.1434
21	RM 23778	9	AACACAGCCTAAAGGTGTTCTGAGC	GCTTCGGCCCTATAGTCTTCTCG	2	0.2950
22	RM 24542	9	ATCCACAAGAGCACCGATGAGG	TGACCTGGTAGTGGTGAGTGTGC	2	0.2701
23	Chr 10-21.2	10	ATGGACACGAACATGCAGAGAGG	AATGATTGCACGGTGTGATGG	2	0.1570
24	RM 24954	10	TGGACAGGTAATCATGTGTCTGC	TTTAGTTGGTGTGCACGTGAGG	2	0.5711
25	JGT 11-15.3	10	GATCGCCCGTTAGCCTTTTCGA	GAACAGTTTTGCTGGCAACAA	3	0.5493
26	RM 25066	10	GTTGTTAGGTGTAGCCGTGTAGG	GTACACCAATAACTGTGGAAGAGC	3	0.6179
27	RM 206	11	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	3	0.6283
28	RM 26784	11	CGTTATTGCGGATGACAGAAACG	GGCATGAGACACAACCAGATCG	2	0.2432
29	RM 19665	12	CGATGTCTTCGAGTCCCTTAACAGG	ACGGTTGGTGATGCTCTTAGGC	2	0.0721
30	RM 28461	12	GAGCTTAGAGGCTGTCCTTGTCC	GCCTGAGTCACGTTTCTTATCAGC	2	0.1178

Table 2: Clustering pattern of 77 genotypes based on molecular data**.**

S. No	Clusters	No. of genotypes	List of genotypes
1	Cluster I	59	Pant Dhan 19, Sukaradhan 1, Bahadur, CR Dhan 300, Dhanrasi, Chittimutyalu, IR 62, DRR Dhan 44, Sameshwari, IR 64, Vivek Dhan 86, Akshaydhan, CR Sugandhdhan, Gontra bidhan 3, Amara, BPT 5204, Jaya, Dubraj, Tarori Basmati, IR 64, PR 113, CR Dhan 201, NDR 8002, FL 478, Pooja, Shobini, Swarna *Sub1*, Karma Mahsuri, Sugandh Samba, Sabita, CSR 10, Varalu, Co 51, Co 43, CSR 23, DRR Dhan 43, Govind, MTU 1010, CST 71, Aadur 1, Swarna, Samba Mahsuri *Sub1*, Jalmagna, Pokkali, Purnedu, Karjat 6, IR 8, IR 52, Lalat, C101A51, Narendra 97, Co50, Salivahana, IR 43, NDR 359, Dinesh, Pushyami, CSR 36, IR 5
2	Cluster II	8	Karjat 7, Shalimar 3, DHMAS 164 2B, Ketekijoha, CR Dhan 500, CR Dhan 202, Tetep, WGL 14
3	Cluster III	3	PR 124, MAS 9461, Kalanamak
4	Cluster IV	2	PB 1, RNR 15048
5	Cluster V	1	Vivek Dhan 62
6	Cluster VI	1	Badshabhog
7	Cluster VII	2	ISM, ADT 49
8	Cluster VIII	1	PB 1121

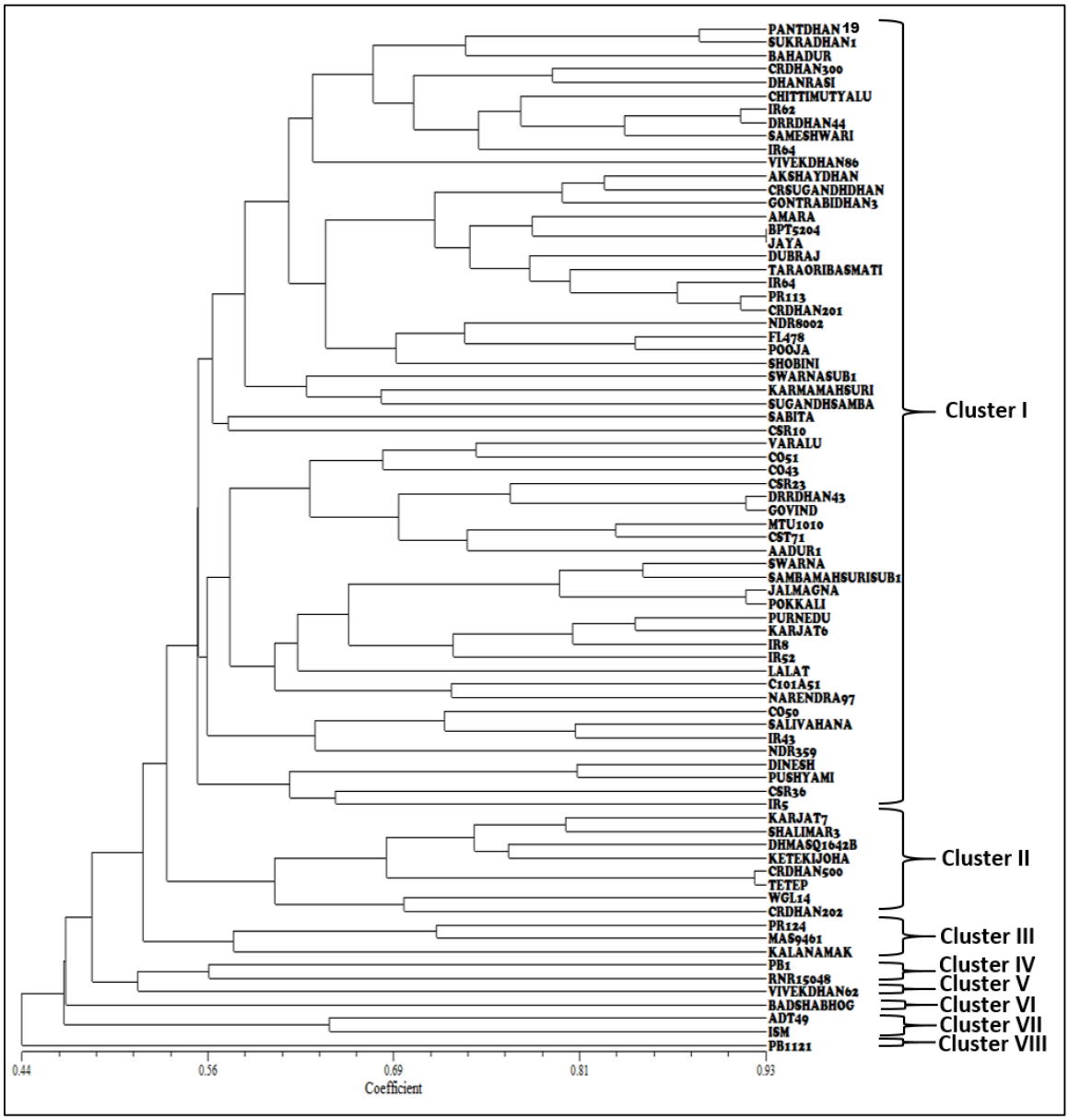

Fig 1: Dendrogram showing the genetic relationships among 77 genotypes of rice using UPGMA clustering method

Conclusion

Exploring the genetic variation among the genotypes plays a major role in varietal improvement program. This is because genetic variations give room for recombinants which are essential in development of new varieties. In this study, molecular diversity analysis using SSR markers has provided clues in the identification of the divergence among rice germplasm. The present study indicated the presence of high amount of diversity among the germplasm lines. Accessions such as Pusa Basmati 1121, Badshabhog and Vivek Dhan 62 singled out with independent clusters. The information about the genetic diversity of these germplasm lines will be useful

for identification and selection of appropriate parents for use in the breeding programmes, gene mapping, identification of parental source and ultimately in marker assisted selection for improvement of rice.

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