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Genetic Divergence Studies in some Indigenous Rice (*Oryza sativa* L.) Germplasm of Northern Dry Zone of Karnataka using Simple Sequence Repeats (SSR)

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Abstract

Simple Sequence Repeat (SSR) among twenty-four rice genotypes, comprising of the land race (1), germplasm (11), INGER breeding lines (8) and Local cultivars (4) was characterized by 14 primers of SSR out of 14 primers, and 13 were polymorphic. The PIC value ranged from 0.076 (RM408) to 0.80 (RM212) with an average of 0.57 per marker. The simple matching similarity coefficient ranged from 0.30 to 0.96. The genotypes were grouped into ten clusters. At the genetic similarity of 85%, allelic variability among the SSR markers was high and sufficient to categorize rice genotypes into ten clusters formed through SSR, data revealed that the genotypes exhibit high genetic diversity since, very much useful in the breeding programme

Keywords: Rice; Genetic diversity; PIC values; SSR markers; Cluster analysis

Introduction

Rice (*Oryza sativa* L.) is the staple food for more than 50% of the world's population. even though high yielding cultivars are available still, rice production and productivity is day by day decreasing due to abrupt climatic aberrations, It has been estimated that world rice production must increase by 40% in 2030 to meet projected demands from population increase and economic development [1] need special attention to understand genetic diversity for a hidden repertoire of novel genes for various abiotic and biotic stress in diverse accessions of rice, owing to rich genetic diversity which is untapped in rice among and between landraces, germplasms, INGER leaving a wide scope for future crop improvement. Genetic diversity in crops has been traditionally evaluating using morphological or physiological traits. The assessment of phenotype may not be a reliable measure of genetic differences as molecular levels and help to identify and develop genetically unique germplasm lines. Since genetic variations based on DNA polymorphism are abundant and independent of environmental factors. specially SSR markers are more popular in rice as they are highly informative, co-dominant, easily analysed, cost-effective, and PCR based, the present investigation was undertaken with an objective of assessment of underlying untapped genetic diversity at molecular level among 24 rice genotypes using SSR markers, could be used for designing effective breeding programs aiming to broaden the genetic bases of commercially grown varieties.

Materials and Methods

Plant materials

Twenty four rice genotypes constituted the experimental material from the agricultural research station, Gangavati. List of rice genotypes **(Table 1),** which includes 11 germplasm, 9 INGER, 4 Cultivars were taken for study in the Kharif season 2011.

Genomic DNA isolation

The DNA was isolated from the 34-day old seedlings leaves by adopting the modified CTAB method [2]. It was found that DNA isolated by a modified CTAB method was of high purity and the yield was also substantial and concentration of DNA was observed by eppendorf bio-photometry. The DNA isolated for all the samples by this method was used for further analysis, The DNA samples were diluted to a concentration of 20 ng/µl with double distilled water for SSR analysis. Fourteen microsatellite primer pairs were selected for the genetic diversity analysis from the list rice genome database that was reported to be trait-specific [3].

SSR amplification

PCR amplification reactions were done in a total volume of 20 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 µM each of deoxynucleotide triphosphate (dNTP), 0.2 µM of each forward and reverse primer, 1 unit Taq DNA polymerase and 20 ng of template DNA. The PCR amplifications were performed using a Bio-Rad thermocycler. The thermal cycler was set at 1 cycle per 5 min at 94°C as an initial hot start and strand separation step. This was followed by 35 cycles of 1 min at 94°C for denaturation, 1 min for annealing temperature depending on the marker used (55-65°C) and 2 min at 72°C for primer elongation. Finally, 1 cycle of 7 min at 72°C was used for the final extension. Amplified products were stored at -20°C until further use. The reproducibility of amplification products was confirmed twice for each of the primers used in this study. After amplification, a 15 µl aliquot of the amplified microsatellite samples was combined with 3 µl of a loading buffer (0.4% bromophenol blue, 0.4% xylene cyanole and 5 ml of glycerol) and was analyzed directly on 3% Agarose gels in 1XTBE buffer (10 mM Tris-Borate, 1 mM EDTA) containing 0.5 µg per ml of ethidium bromide. A 25 bp DNA ladder was used as a size marker to compare the molecular weights of amplified products. After electrophoresis, the gels were documented using an Alpha Gel Documentation System.

Table 1: The list of rice genotypes used for molecular diversity study.

S. No	Type of material	Genotypes
1	GERMPLASM	GNV-GP-11-4082, GNV-GP-11-4029, GNV-GP-11-4017, GNV-GP-11-4048, GNV-GP-11-4083, GNV-GP-11-4087, GNV-GP-11-4005, GNV-GP-11-4050, GNV-GP-11-4027, GNV-GP-11-4047
2	LOCAL CULTIVARS	GGV-05-01, BPT-5204, P-27PO4, IET-19251, GNV-GP-11-4014,
3	LAND RACE	BELINELLU
4	INGER*	AG-7, PSBRC-18, OM6162, A-69-1, BR-6926-3-1-4-5-4,PK-8573-4-1, CB-01-001, CB-01-508,

* International network for genetic evaluation of rice

Note: GNV-GP-11 (Gangavathi germplasm 2011)

Table 2: List of markers, primer sequences and their respective chromosome numbers used for molecular analysis [9].

S. No	Locus name	Trait	Chr. No	Sequence	Annealing temperature (°C)	Product size (µl)
1	RM 212	Grain length, Deep root mass [4]	1	F 5' CCACTTTCAGCTACTACCAG 3' R5'CACCCATTTGTCTCTCATTATG3'	55	136
2	RM 315	Plant height, and panicle length [5]	1	F 5' GAGGTACTTCCTCCGTTTCAC3' R5'AGTCAGCTCACTTGCAGTG3'	55	133
3	RM 242	Days to heading, spikelet number [6]	9	F 5' GAGCCAAATAAGATCGCTGA3' R5'TGCAAGCAGCAGATTTAGTG3'	55	225
4	RM 1	Grain yield/plant [7]	1	F 5' GCGAAAACACAATGCAAAAA3' R5'GCGTTGGTTGGACCTGAC3'	55	113
5	RM 11	Days to maturity, seed number [7]	7	F 5' TCTCCTCTTCCCCCGATC3' R5'ATAGCGGGCGAGGCTTAG3'	55	140
6	RM 52	No. of tillers, zinc content [8]	8	F 5' GAAACCACCACACCTCACCG3' R5'CCGTAGACCTTCTTGAAGTAG3'	55	151
7	RM 408	Days to maturity [5]	8	F 5' CAACGAGCTAACTTCCGTCC3' R5'ACTGCTACTTGGGTAGCTGACC3'	55	128
8	RM 536	Panicle number [5]	11	F 5' TCTCTCCTCTTGTTTGGCTC3' R5ACACACCAACACGACCACAC3'	55	243
9	RM 7	Spikelet number, grain length, [7]	3	F 5' TTCGCCATGAAGTCTCTCG3' R5CCTCCCATCATTTCGTTGTT3'	55	180

10	RM 224	Panicle length, 1000-grain weight [4]	11	F 5' ATCGATCGATCTTCACGAGG3' R5TGCTATAAAAGGCATTCGGG3'	55	157
11	RM 263	Grain yield, harvesting index [4]	2	F 5' CCCAGGCTAGCTCATGAACC3' R5GCTACGTTTGAGCTACCACG3'	55	199
12	RM 190	Panicle length [8]	6	*Oryza sativa* Indica Group Genomic DNA [5317]	55	124
13	RM 13	Grain yield, BPHRS [7]	5	F 5' TCCAACATGGCAAGAGAGAG3' R5GGTGGCATTCGATTCCAG3'	55	141
14	RM 201	Seed set per cent, leaf area [4]	9	F 5' CTCGTTTATTACCTACAGTACC3' R5CTACCTCCTTTCTAGACCGATA3'	55	158

Data analysis

Only clear unambiguous polymorphic bands were scored visually for their presence or absence. The scores were obtained in the form of a matrix with '1' and '0', which indicate the presence and for the absence of bands in each genotype respectively. The binary data scored was used to construct Dendrogram. Polymorphism Information Content (PIC) or expected heterozygosity scores for each SSR marker was calculated by software PowerMarker V3.0 [10].

The pair-wise comparisons of the genotypes based on the proportion of unique and shared amplification products (alleles) were used to measure the genetic similarity by Dice coefficients. The Dice coefficients were employed by using the SIMQUAL sub-program in the similarity routine of software NTSYS-pc version 2.2 software package [11]. The estimation of genetic similarity was calculated by following the method described elsewhere [12]. The resultant similarity matrix data was employed to construct a dendrogram by using Sequential Agglomerative Hierarchical Nesting (SAHN) based on unweighted pair group method with an arithmetic average (UPGMA) [13] to infer genetic relationships and phylogeny among cultivars computation were done by using NTSYS-pc version 2.2 software package [11].

Results

A considerable amount of variation was found among different genotypes. The used markers showed several bands; the level of polymorphism among the rice genotypes was evaluated by calculating allele number and PIC values for each of 14 SSR loci evaluated. Each marker pairs differed significantly in their ability to determine variability among the genotypes among the polymorphic markers, 13 produced 2 alleles each, 1 marker gave 3 alleles, a total of 29 alleles were detected across 24 genotypes of which 27 were polymorphic using 14 SSR markers **(Table 2).**

The polymorphism percentage was 96.96. The number of alleles detected per primer pair ranged from 1 to 3 with an average of 2.074 **(Table 3)** the maximum number of amplified products was observed in the profiles of the primer RM212 and RM315. The minimum number of amplified products was observed in the profiles of primer RM408 and RM11. The primers RM408 and RM11 were proved to be less polymorphic and rest were polymorphic. The SSR product size ranged from 148 to 302 bp. The PIC value is a reflection of allele diversity and frequency among the varieties, the PIC value of each marker can be evaluated on the basis of its alleles and it varied greatly for all SSR loci tested. The level of polymorphism among the 24 varieties was evaluated by calculating PIC values for each of the 14 SSR loci. The PIC values varied widely among loci and ranged from 0.076 (RM408) to 0.80 (RM212) with an average of 0.57 per marker **(Figures 1 and 2).** PIC values showed a significantly positive linear correlation with numbers of alleles at SSR loci.

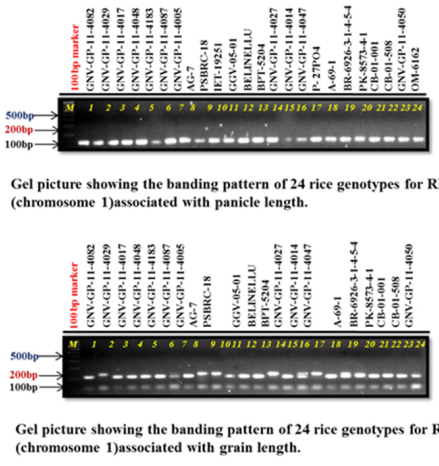

Figure 1: SSR marker profile of rice genotypes generated by the primer RM212.

SSR derived data were subjected to analyse the genetic similarity, similarity matrix was used to determine the level of genetic relatedness among the genotypes studied, The binary data from the polymorphic primers were used for computing simple matching similarity indices. The similarity index values

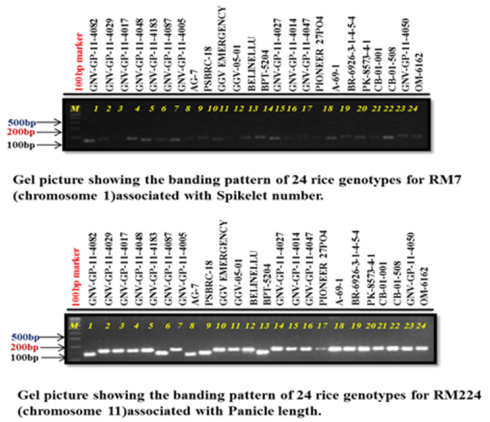

Figure 2: SSR marker profile of rice genotypes generated by the primer RM224.

Table 3: Details of markers, allelic frequency, PIC value and genetic diversity of the SSR markers across 24 cultivars.

Marker	Number of alleles	Number of p polymorphic alleles	Polymorphism (%)	PIC
RM212	2	2	100	0.8074
RM315	2	2	100	0.7663
RM242	2	2	100	0.7481
RM1	2	2	100	0.3536
RM11	3	1	80.00	0.2212
RM152	2	2	100	0.5609
RM408	2	2	100	0.0767
RM536	2	2	100	0.4881
RM7	2	2	100	0.6923
RM224	2	2	100	0.5968
RM263	2	2	100	0.6371
RM190	2	2	100	0.7649
RM13	2	2	100	0.3694
RM201	2	2	100	0.5331

obtained for each pair wise comparison among the 24 genotypes the similarity coefficients based on 29 alleles of 14 SSR markers ranged from 0.30 to 0.96 Among the 24 genotypes the highest similarity index (0.96) was observed between GNV-GP-11-4017 and GGV-05-01 and the lowest similarity index (0.30) was observed between AG-7 and BR-6926-3-1-4-5-4.

The similarity values obtained for each pair wise comparison of SSR markers among the 24 rice genotypes were used to construct dendrogram based on simple matching coefficient indices **(Table 4).** The 24 genotypes formed 10 clusters at nearly 85% similarity levels. Among the different clusters, the cluster size varied from 10 (Cluster IV) to 1 (Clusters VI, XI, and X).

The list of all the 10 clusters along with the genotypes included is presented in **(Figure 3)** the cluster IV was highly heterogeneous. The cluster I consisted of GNV-GP-11-4082 and GNV-GP-11-4087. The cluster II consisted of GNV-GP-11-4005, the cluster III consisted of GNV-GP-11-4017, GGV-05-01, CB-01-001, GNV-GP-11-4047, and BR-6926-3-1-4-5. The cluster IV consisted of genotypes GNV-GP-11-4048, GNV-GP-11-4014, GNV-GP-11-4027, OM 6162, GNV-GP-11-4083 and P-27PO4. The cluster V consisted of genotypes BELINELLU, PK-8573-4-1 A-69-1, CB-01-508, and GNV-GP-11-4050. The cluster VI consisted of genotypes GNV-GP-11-4029, and G-EMERGENCY (IET19251). The cluster VII consisted of genotypes, GNV-GP-11-4027. The cluster VIII consisted of genotypes, PSBRC-18. The cluster IX consisted of genotypes BPT-5204. The cluster X consisted of genotypes AG-7.

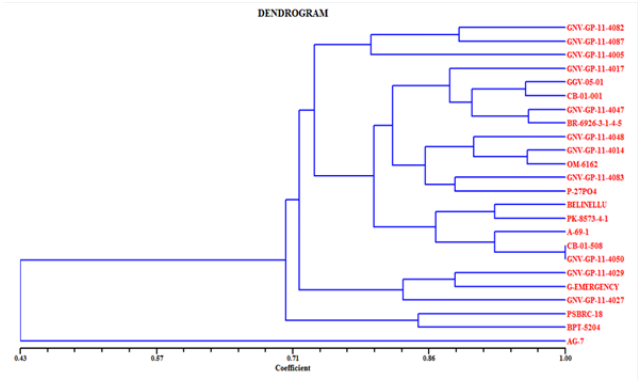

Figure 3: Dendrogram of rice genotypes based on the SSR marker data.

Discussion

The SSR is amongst the most widely used DNA markers types for many purposes genotypes identification, characterization of germplasm, genetic diversity analysis, tagging of genes of complex traits, comparative genome analysis for novel traits due ease analysis. The application of these SSR markers to investigate the genotypic variations among different genotypes by other researchers [14-16] The implication of these SSR markers to determine the genetic diversity of 24 genotypes. The results indicated a high level of

genetic variation in the genotypes used, the results based on 14 SSR markers, the analysis showed a clear division of genotypes, the number of alleles detected by SSR microsatellite markers varied from 1 to 3 with an average 2.074 alleles per locus. The numbers of alleles observed in the present study correspond well to some earlier bibliographic reviews on the Indian rice varieties [17,18]. The level of polymorphism determined by the PIC value was quite high and varied (range 0.076 to 0.80 with an average of 0.57 per marker) considerably among SSR loci. Similarity coefficients among various cultivars ranged from 0.30 to 0.96 in present investigations. Saini et al. [19] reported almost similar values of similarity co-efficient among 18 basmati and non-basmati varieties using molecular markers.

Cluster analysis based on similarity coefficients placed 24 rice genotypes into 10 clusters. Similar studies conducted by Nagaraju et al. [15] and Saini et al. [19] using SSR markers, long-grain basmati cultivars were grouped together, whereas the other short-grained non-aromatic rice fell into different groups. These authors also reported that traditional and evolved basmati varieties shared a high degree of similarity using SSR markers.

Though the number of germplasm genotypes analysed in our study, our results show a clear distinction between closely related germplasm and other rice genotypes. The characterization and quantification of genetic diversity within closely related germplasm is a major aim, it is essential for rational use of genetic resources, the analysis of genetic variation among the germplasm is fundamental interest to plant breeders, contributes immensely to the selection, monitoring of germplasm and prediction of genetic gains. Ravi et al. [20] could also generate unique SSR profiles in rice by using a few primers that covered all 12 chromosomes. In the present study, the average alleles per locus were 2.074 indicates that a greater magnitude of diversity among the plant materials. Many studies have also reported significant differences in allelic diversity among various microsatellite loci [8,20,21]. The alleles revealed by markers showed a high degree of polymorphism, with as many as 12 producing 96.96% of bands were polymorphic and suggested that the genotypes selected for this study had enough genetic diversity. The markers showed an average PIC value of 0.57, which confirms that SSR markers used in this study were highly informative, because PIC values higher than 0.5 indicate high polymorphism since, useful in genetic studies and distinguishing the polymorphism rate of a marker at a specific locus [21].

The mean PIC value observed in this study was 0.57 similar results were reported in an earlier study among rice germplasm [22]. Study indicates that the genotypes used in the present study were more diverse due to differences in origin, ecotype. SSR markers exhibit high PIC values because of their co-dominant expression and multiallelic moreover,14 microsatellite markers were able to discriminate between the landraces, cultivars, germplasm and demonstrated a maximum genetic similarity value of 0.96 between the GNV-GP-11-4017 and GGV-05-01. The minimum genetic similarity value of 0.30 was observed between the was observed between AG-7 and BR-6926-3-1-4-5-4. Studies of Panaud et al. [7] using SSR markers in rice described similarly high genetic similarity among rice genotypes. UPGMA cluster analysis of the SSR based genetic similarity matrix resulted in the classification of germplasm, landraces, cultivars, and INGER since SSR markers detect finer levels of variations among closely related lines. The dendrogram **(Figure 3)** resulting from UPGMA analysis could reveal the allelic richness of 10 clusters for various sizes at a similarity coefficient level of 0.85%. Cluster V was the largest which includes 6 genotypes and while clusters III and IV included 5 genotypes each **(Table 4).**

Table 4: Cluster compositions of rice genotypes for SSR markers.

Cluster no.	No. of genotypes	List of genotypes
I	2	GNV-GP-11-4082, GNV-GP-11-4087
II	1	GNV-GP-11-4005
III	5	GNV-GP-11-4017, GGV-05-01, CB-01-001, GNV-GP-11-4047, BR-6926-3-1-4-5
IV	5	GNV-GP-11-4048,GNV-GP-11-4014, GNV-GP-11-4027, OM 6162, GNV-GP-11-4083, P-27PO4
V	5	BELINELLU, PK-8573-4-1 A-69-1, CB-01-508, GNV-GP-11-4050,
VI	2	GNV-GP-11-4029, G-EMERGENCY(IET19251)
VII	1	GNV-GP-11-4027
VIII	1	PSBRC-18
IX	1	BPT-5204
X	1	AG-7

Conclusion

In conclusion, the allelic diversity revealed by 14 SSR primers was sufficient enough to distinguish between the germplasm, cultivars, land race and INGER. The allelic variation was lower within the cultivars than germplasm, landraces and, indicating the chance to exploit distant relatives to broaden the genetic base of rice genotypes.

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