Molecular Profiling of Parents of Some Released Rice Hybrids Using SSR Markers

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SSR markers targeting (GATA)n motifs are known to be highly polymorphic and useful in many organisms. Twenty eight germplasm of rice (*Oryza sativa* L.) involved as parents of some of the released hybrids were subjected to polymorphism based on JGT-SSRs analyses to asses the prevalence of genetic diversity. A total of 14 primers of SSRs amplified 37 polymorphic loci. The polymorphic information content ranged from 0.535 (JGT12-8.6) to 0.929 (JGT1-16.2). Prevalence of genetic diversity was revealed by (GATA)n SSRs as range of similarity coefficient value of 0.31 to 1. The parents were grouped by subjecting the similarity values to UPGMA based SAHN clustering. Study revealed the existence of sufficient genetic variation at DNA level in the genotypes being used as parental lines. The results suggested that a relatively less number of (GATA)n microsatellite markers can be used for the reliable estimation of genetic diversity and identification of duplicates.

Key Words: Genotypes, JGT-SSRs, Molecular diversity, Rice

Introduction

An accurate classification of parental lines into heterotic groups is essential to facilitate selection of parents and predicting the performance of F_1 hybrids. Recent studies of genetic distance analysis of rice cultivars have focused on molecular genetic markers (Naghia *et al.,* 2002). Markers based on differences in DNA sequences between individuals generally detected more polymorphism than morphological and protein based markers. Among the series of DNA markers that can be used for detecting polymorphism, SSR markers are the important class. SSR-PCR technique is a simple and quick method that combines most of the advantages of microsatellites and amplified fragment length polymorphism to the universality of Randomly Amplified Polymorphism. Most of the microsatellite loci studied so far mainly contain dinucleotide and trinucleotide motifs while very few tetranucleotide motifs have been identified (http://www.gramene.org and McCouch *et al.,* 2002). With the availability of whole genome sequence of rice in the public domain, it is now possible to identify the motifs of interest through in silico approaches and develop PCR-based markers. Studies have revealed that (GATA)n containing sequences are abundant in many eukaryotic genomes (Singh, 1995). The utility of microsatellite markers targeting (GATA)n motifs in paternity testing, genetic counseling and individual identification has been well demonstrated (Pena *et al*. 1994). A few preliminary studies in pearl millet (Chowdari *et al*., 1998), rice (Davierwala *et al*. 2001), sunflower (Mosges and Friedtu, 1994) and tomato (Rao *et al*. 2006) have highlighted the potential of (GATA)n motifs in determining the genetic variability among cultivars. However, application of these markers for revealing genetic diversity is at starting stage. The objective of the present study is to detect genetic diversity among parental lines using the polymorphism developed by (GATA)n SSR markers.

Materials and Methods

Twenty-eight genotypes [five cytoplasmic male sterile (CMS) lines and 23 restorer (R) lines representing parental lines of experimental rice hybrids] given in Table 1 were used in the present study. The leaf material of all the twenty eight genotypes were collected from Research Farm, Directorate of Rice Research (DRR), Hyderabad.

Genomic DNA from the leaf samples (25 days old seedlings) was isolated using the procedure of Zheng *et al.* (1995) with some modifications. The isolated genomic DNA was quantified using UV Spectrophotometer (Beckmann, DU 650). The isolated genomic DNA was verified for size, intactness, homogeneity and purity. Agarose 0.8% was melted to dissolve completely and

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Table 1. The lines representing parental lines of hybrids used in molecular divergence

S.No	Genotypes	Varietal Type		Genotypes	Varietal Type
1	IR 79156A	A-Line	15	1096	R-Line
2	APMS 6A	A-Line	16	IBL57	R-Line
3	PUSA 5A	A-Line	17	619-2	R-Line
4	CRMS 32A	A-Line	18	EPLT109	R-Line
5	IR 58025A	A-Line	19	SC5 2-2-1	R-Line
6	KMR 3 R	R-Line	20	SC5-9-3	R-Line
7	BR827-35	R-Line	21	SG27-77	R-Line
8	1005	R-Line	22	SG26-120	R-Line
9	611-1	R-Line	23	118	R-Line
10	612-1	R-Line	24	124	R-Line
11	GQ-25	R-Line	25	517	R-Line
12	GQ37-1	R-Line	26	R43	R-Line
13	GQ70	R-Line	27	R55	R-Line
14	GQ120	R-Line	28	R60	R-Line

the gel was casted using the gel casting plate. Genomic DNA samples of 28 rice genotypes were loaded in each well along with 2 µl of tracking dye and run at a constant voltage of 90 volts. The gel was stained using ethidium bromide and placed over an UV trans-illuminator and viewed at 300 nm. The bands appeared as orange-coloured intact bands owing to the florescence of ethidium bromide and the gel was photographed using ALPHA IMAGER Gel documentation System (Alpha innotech, USA).

Quantity and quality of genomic DNA

DNA was isolated from approximately 2 cm leaf bit and quantified. Approximately 3-5 µg of DNA was isolated per sample. The DNA was found to be of high molecular weight, intact and devoid of RNA.

Selection of SSR Markers Targeting $(GATA)_n$ Motifs

A total of 24 $(GATA)_n$ motifs with maximum possible repeat lengths were selected and primers (Table 2) were designed using the software Fast PCR (Kalendar, 2009). It was ensured that 1-2 markers were designed for each chromosome. PCR primer pairs for the selected SSR markers targeting $(GATA)_n$ motifs were synthesized by M/s Integrated DNA technologies, USA. The list of JGT markers used for the present study is given in Table 2.

Table 2. Details of 24 (GATA)n based SSR markers used in the present study

Primer name	Primer (5'-3')	Motif	Location of GATA motif
JGT01-16.2F	tgttggctgaatgattgatggataga	(GATA)11	Upstream
JGT01-16.2R	ggacctatgtggcacgaatttgaa		
JGT02-35.2F	cggggcgaatcgaggtcag	(GATA)14	Exon
JGT02-35.2R	tcgcggtggattagatgcctc		
JGT03-0.01F	ccgatgcaccaacaccctcac	(GATA)11	Intron
JGT03-0.01R	gacgcatggtgagtttggagtgat		
JGT03-26.8F	gagcgtttgtagtaagtttcatggac	(GATA)21	Upstream
JGT03-26.8R	ggcccaacccaaacacaaat		
JGT03-36.1F	gcggcaacacgaccagctt	(GATA)10	Upstream
JGT03-36.1R	catcctgagttttgagaagcaccata		
JGT04-11.5F	agggctcgtagaaagtgtccaattag	(GATA)13	Upstream
JGT04-11.5R	ccgctgggtattgcctaactg		
JGT04-18.1F	cgtggcggtatatgagcgtttgta	(GATA)21	Intron
JGT04-18.1R	agcgggcctcatctccatagtc		
JGT04-28.5F	ttgaatagatcgcaccgttgac	(GATA)12	Upstream
JGT04-28.5R	aaaagggcagctacgtctgagc		
JGT05-7.8F	acggtgagattaggtattgcca	(GATA)10	Intergenic
JGT05-7.8R	agttcggacaaggggagtact		
JGT06-6.8F	gagcgtttgtaggaagtttcatggat	(GATA)9	Intergenic
JGT06-6.8R	ggacaaccacaagcacaccactct		
JGT06-6.9F	tggagaggagtggatgcgtaccta	(GATA)7	Intron
JGT06-6.9R	tctattggagatgaaccaaccacc		
JGT07-17.5F	cattggatgtatagccgatagctc	(GATA)9	Intergenic
JGT07-17.5R	ttgagatggtgagcggatagccac		
JGT07-22.8F	tggcgatctaggagcgtctgt	(GATA)27	Intergenic
JGT07-22.8R	tgtaaacatttcaaaagggcactaa		
JGT07-25.2F	agctgttgtgcatgatgttcgta	(GATA)10	Intron
JGT07-25.2R	ggcgaggggtacaaggactt		
JGT08-16.7F	gaaagaatttgttcccacgacctc	(GATA)8	Intron
JGT08-16.7R	gctacaattgcatgctggatgtcg		
JGT09-4.8F	cgggatactaacagcaagcaaat	(GATA)8	Upstream
JGT09-4.8R	tctcgcaccactaacagggaaca		
JGT10-5.7F	tatggaagcagcaagtcggag	(GATA)9	Intergenic
JGT10-5.7R	gttgatccttcacgaccaccaa		
JGT11-7.8F	cggtttaggagcgcttgtagga	(GATA)9	Intergenic
JGT11-7.8R	atcccttgtatgccactgaaaattg		
JGT11-9.9F	tctggaatgtgtccttggggga	(GATA)9	Upstream
JGT11-9.9R	ctaggtttaggtgaccaacagtc		
JGT11-15.2F	gatcgcccgttagccttttcga	(GATA)14	Intron
JGT11-15.2R	gaacagttttgctggcaacaa		
JGT11-16.3F	ggcggcgtattagcgttgta	(GATA)13	Intron
JGT11-16.3R	aggttctagcccatgttaaatcttct		
JGT11-22.7F	ggcggcataggagtgtttgtag	(GATA)12	Upstream
JGT11-22.7R	cgcggtggtcgggatgag		
JGT12-8.6F	atggcagcgtaggagcgtttgtag	(GATA)13	Intron
JGT12-8.6R	aacgtacctaggcagcaaaattct		
JGT12-7.7F	gagcgttttgaggaagtttaatggac	(GATA)8	Intron
JGT12-7.7R	ggcaccgtttgatttagattattacc		

Polymerase chain reaction (PCR) amplification

The genomic DNA of the 28 rice genotypes isolated as described earlier were subjected to PCR amplification as per the procedure described by Panaud *et al.* (1996). Briefly, 10 µl reaction mixture contained 0.2 µM of each primer (both forward and reverse primers), 200 µM of deoxyribonucleotides, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, 40-50 ng of DNA and 0.5 unit of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). The following thermal profile was followed: Initial denaturation at 94°C for 5 minutes and subsequent 35 cycles, each with denaturation for 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and primer elongation at 72°C for 1 min. The final elongation was performed at 72°C for 7 min.

Agarose gel electrophoresis of PCR products

PCR amplified products were resolved in 4% super fine resolution (SFR) agarose gels in 0.5X TBE buffer at 200V for 3.5 hrs using Submarine Horizontal Electrophoresis Unit (CBS Scientific, USA). Before loading, the PCR amplified products were mixed with 1/6th volume of gel loading dye (40% sucrose; 0.25% bromophenol blue). The sizes of amplified fragments were determined by comparing with 50 bp ladder (MBI Fermentas). The gels were stained in Ethidium Bromide (10mg/ml) for 3 min, destained in distilled water for another 2 min, placed over the UV-transilluminator and documented at 300 nm using ALPHA IMAGER gel documentation system (M/s Alpha innotech).

Data analysis

Scoring

Qualitative multistate traits that depict an array of characters were converted into binary characters (Sneath and Sokal, 1973) based on the variations present. Only the clear and unambiguous bands of SSR markers were scored. Markers were scored for the presence and absence of the corresponding band among the genotypes. The score 1 and 0 indicate the presence and absence of the bands respectively. A data matrix comprising of '1' and '0' were formed depending upon the character and this data matrix was subjected to further analysis.

Marker polymorphism

To measure the informativeness of the markers, the polymorphism information content (PIC) for each SSR marker was calculated according to the formula:

$PIC=1-SPi^2 - SSPi^2\ Pj^2$

Where, 'i' is the total number of alleles detected for SSR marker and 'Pi' is the frequency of the ith allele in the set of 28 genotypes investigated and j=i+1. This formula gives us an indicator of how many alleles a certain marker has, and how much these alleles divide evenly. The PIC value was calculated using the online software– 'Polymorphism Information Content Calculator' available at *www.agri.huji.ac.*

Cluster analysis

The binary data matrix is subjected to cluster analysis. Sequential Agglomerative Hierarchial Non-overlapping (SAHN) clustering was performed on Squared Euclidean distance matrix and similarity matrix using Jacquard's coefficient utilizing the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method. Data analysis was done using the software NTSYSpc version 2.02 (Rohlf, 2002).

Results and Discussion

PCR Amplification Status of GATA Specific SSR Markers

Out of the 24 GATA specific SSR markers used in the present study, 5 failed to give any amplification. Out of the remaining 19 GATA specific markers amplified (Table 3), 14 were found to be polymorphic and generated a total of 37 alleles in the 28 genotypes studied. The number of alleles per SSR marker ranged from 2-4 with an average value of 2.64. The polymorphic information content (PIC), which is a measure of allelic diversity at a locus ranged from 0.535 (JGT12-8.6) to 0.929 (JGT1-16.2). Information regarding the polymorphism status of the GATA specific SSR markers is given in Table 3 along with their PIC values. Gel images of the GATA specific (JGT 3-26.8 and JGT 11-16.2) SSR markers that exhibited good polymorphism are presented in Fig.1.

Table 3. Allelic distribution of polymorphic JGT (GATA) SSR markers among 28 genotypes

Marker Name	No. of Alleles	Allele1	Allele2	Allele3	Allele4	Polymorphic information of content (PIC)	Polymorphic alleles
JGT1-16.2	3	3	5	20	-	0.929	3
JGT2-35.2	2	25	3	-	-	0.596	2
JGT3-00.1	4	7	2	16	3	0.899	4
JGT3-26.8	3	8	11	9	-	0.886	3
JGT3-36.1	3	19	4	5	-	0.833	3
JGT4-11.5	3	1	7	20	-	0.634	3
JGT4-18.1	2	24	4	-	-	0.634	2
JGT6-6.8	3	4	2	22	-	0.730	3
JGT6-6.9	3	4	23	1	-	0.768	3
JGT7-25.2	2	26	2	-	-	0.566	2
JGT9-4.8	2	12	16	-	—	0.745	2
JGT11-15.2	2	12	16	-	-	0.745	2
JGT11-16.3	3	17	7	4	-	0.877	3
JGT12-8.6	2	27	1	-	-	0.535	2

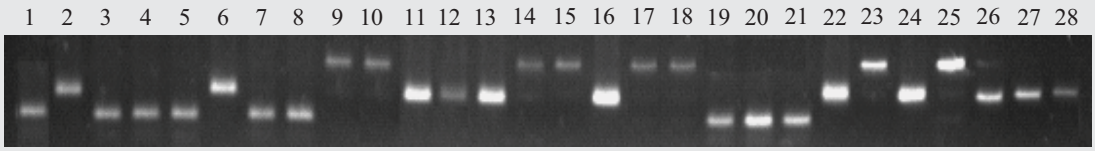
Allelic distribution of GATA specific SSR markers

Each amplicon (band) was considered as an allele in SSR analysis. The marker JGT3-0.01 generated a maximum of four alleles, while JGT1-16.2, JGT3-26.8, JGT3-36.1, JGT4-11.5, JGT6-6.8, JGT6-6.9 and JGT11-16.3 generated 3 alleles. Six of the GATA Specific markers (JGT2-35.2, JGT4-18.1, JGT7-25.2, JGT9-4.8, JGT11-15.2 and JGT12-8.6) generated 2 alleles, while JGT4-28.5, JGT5-7.8, JGT11-7.8, JGT11-9.9 and JGT11-22.7 showed only one allele (monomorphic) among the genotypes. The allelic distribution of the 14 GATA Specific markers among the 28 genotypes is given in Table.3.

Cluster analysis

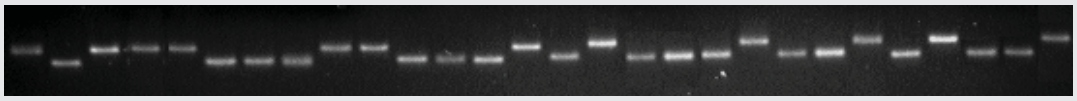
The cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from GATA specific markers to generate a dendrogram of the 28 genotypes with UPGMA algorithm (Fig. 2). The similarity coefficient ranged from 0.28 to 1.00. The dendrogram

Plate 1: Gel photograph showing the amplification pattern with JGT3_26.8 Primer



1 - IR79156A
2 - APMS6A
3 - PUSA5A
4 - CRMS32A
5 - IR58025A
6 - KMR3R
7 - BR827 -35
8 - 1005
9 - 611 -1
10 - 612 -1
11 - GQ -25
12 - GQ37 -1
13 - GQ70
14 - GQ120
15 - 1096
16 - IBL57
17 - 619 -2
18 - EPLT109
19 - SC5 2 -2-1
20 - SC5 -9-3
21 - SG27 -77
22 - SG26 -120
23 - 118
24 - 124
25 - 517
26 - IR43
27 - IR55
28 - IR60

Plate 2: Gel photograph showing the amplification pattern with JGT11_16.2 Primer



1 - IR79156A
2 - APMS6A
3 - PUSA5A
4 - CRMS32A
5 - IR58025A
6 - KMR3R
7 - BR827 -35
8 - 1005
9 - 611 -1
10 - 612 -1
11 - GQ -25
12 - GQ37 -1
13 - GQ70
14 - GQ120
15 - 1096
16 - IBL57
17 - 619 -2
18 - EPLT109
19 - SC5 2 -2-1
20 - SC5 -9-3
21 - SG27 -77
22 - SG26 -120
23 - 118
24 - 124
25 - 517
26 - IR43
27 - IR55
28 - IR60

Fig. 1. Amplication pattern of (GATA) n specific SSR markers among 28 rice genotypes

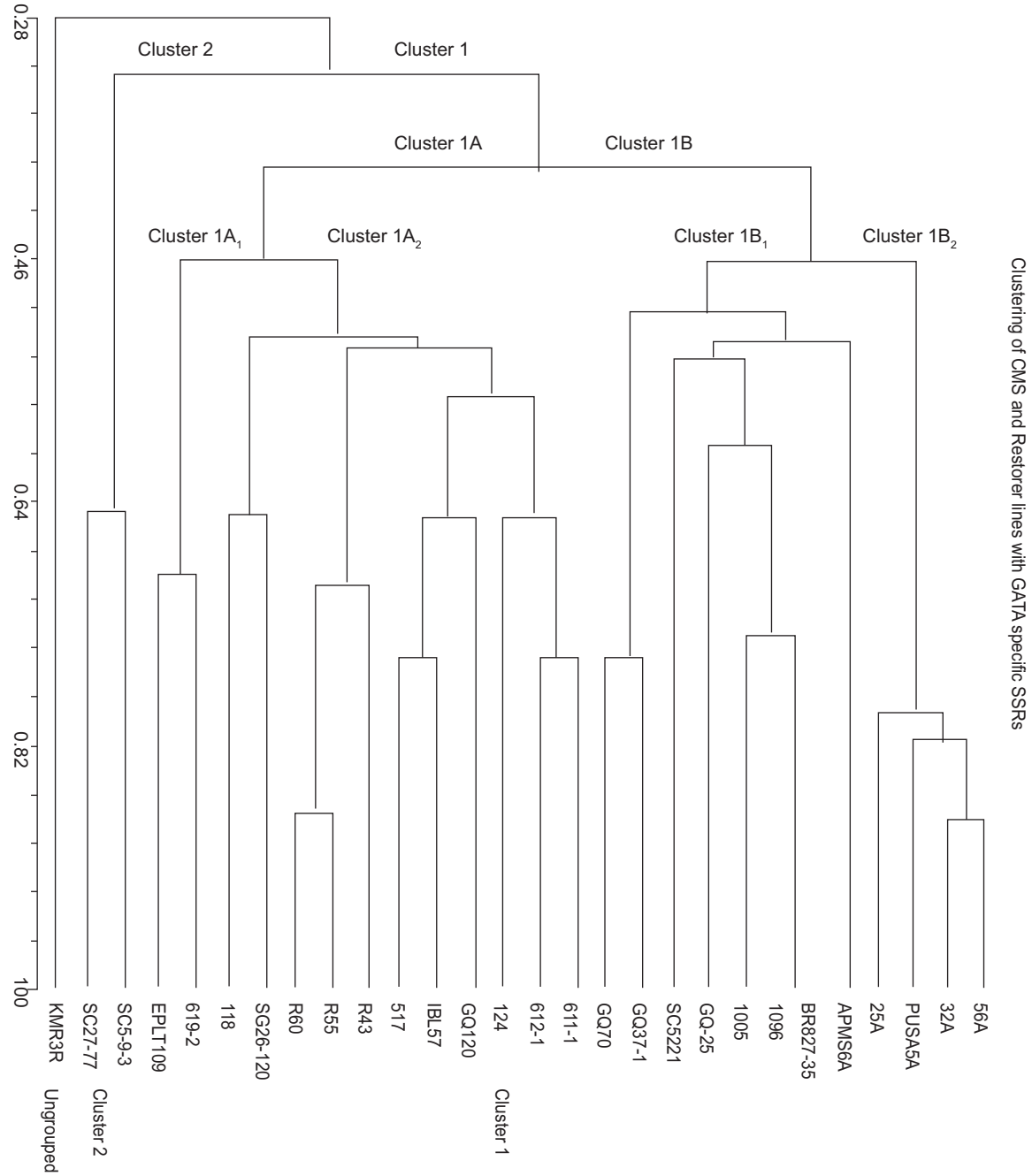


Fig. 2. Clustering of CMS and restorer lines with GATA specific SSRs

showed the grouping pattern of the 28 rice genotypes analyzed. All the 27 genotypes were grouped into two major clusters with 31.6% similarity among them, while one genotype KMR3 remain ungrouped. Two genotypes SG27-77and SC5 9-3 were clustered in cluster 2, whereas cluster 1 divided in to cluster1A and cluster 1B. The cluster 1A was further divided in to cluster $1A_1$ and cluster $1A_2$. In this study, the parents formed distinct subgroups from main clusters, a phenomenon which had been noticed in other studies also (Garris *et al.,* 2005; Jain *et al.,* 2004). The cluster $1A_1$ was with two genotypes EPLT 109 and 619-2, while eleven genotypes *viz.,* 118, SG 26-120, R 60, R 55, R 45, 517, IBL-57, GQ-120, 124, 612-1 and 611-1 were clustered in the cluster $1A_2$. The cluster 1B was divided in to cluster $1B_1$ and cluster $1B_2$. The sub cluster $1B_1$ had GQ-70, GQ37-1, SC5 2-2-1, GQ-25, 1005, 1096, BR 827-35 and APMS 6A, whereas four CMS lines *viz.,* IR 58025A,

PUSA5A, CRMS32A and IR 79156A were clustered under the sub cluster $1B_2$. This pattern of grouping in to sub clusters corresponds to that of Glaszmann (1987) and Garris *et al*. (2005), thus, highlighting the potential of (GATA)n motif specific microsatellite markers in genetic diversity studies.

The average similarity index based on Jaccard coefficient was 0.316, indicating a high level of diversity among the lines included in the study. The similarity coefficients ranged from (0.316) to (1.00). The representatives of similarity coefficient among the 28 rice accessions are presented in Fig. 2. The dendrogram classified rice germplasm in to two major clusters (cluster 1 and cluster 2) with 25 and 2 rice genotypes, respectively. Among the restorer parents with different names the accessions BR827-35 and 1096 with 100% similarity coefficient were collected from different sources, which may be considered as possible duplicates. Considering the grouping pattern and place of collection, GQ 37-1 and GQ 70 with 76.9% similarity;1096 and 1005 with 73% similarity; 611 and 612-1 with 77.2% similarity; 619-2 and EPLT -109 69.7% similarity; SC59-3 and SG 27-77 66% similarity may be considered as suspected duplicates. Out of the three IRRI restorer accessions R 55 and R 60 collected from same locations were grouped together with 86.8% similarity, these two accessions can be considered as potential duplicates and R 45 can be considered as different based on grouping pattern.this is in consonance with earlier results. Olufowote *et al.* (1997) reported that the other type of DNA markers to that of RAPD gaining momentum in its usage in rice is microsatellites or SSR. The SSR markers were employed to determine the degree of relatedness and to detect duplications and seed mixtures

However, SC5 2-2-1, SG 26-120 and SC5 9-3 collected from the same source grouped differently revealing some genetic differences between them. Similarly four genotypes (GQ-25, GQ 37-1, GQ-70 and GQ-120) collected from DRR were also grouped differently, revealing genetic differences. On the other hand accessions with same name were grouped differently like 1096 grouped with BR827-35, IBL-57 with 517, SG 26-120 with 118 and 619-2 with EPLT-109, though the place of collection was same for these collections, they are genetically different. Among the 5 CMS lines included in the study, 25A and 56A were grouped in to two clusters indicating the genetic differences. Interestingly, two CMS lines 56A (IRRI, Philliphines) and 32A (CRRI, Karnal) from different places were grouped under same cluster with 84.5% similarity. Among the 5 CMS lines APMS 6A from ANGRAU, Maruteru is different from rest of the CMS lines indicating sufficient genetic variation in producing different heterotic hybrids with different R line combinations.

The present study revealed the existence of sufficient genetic variation at DNA level in the genotypes being used as parental lines based on (GATA)n motifs at DRR, Hyderabad. Being highly polymorphic with robust amplification, the (GATA)n motif based microsatellite markers can be used for genetic diversity analysis and cultivar identification (Ni *et al*., 2002). The average number of alleles and PIC value recorded for GATA markers is slightly lower than that recorded for genomic microsatellite markers in rice (Coburn *et al.*, 2002; Ni *et al.* 2002). Despite this, it is interesting to note that (GATA)n motif specific markers exhibited good allelic diversity within cultivar groups in the present study. This diversity information will help the breeder to select diverse genotypes based on the variation at DNA level to be used in the crossing programme for realization of heterosis, identification of donor parents for useful agronomic attributes and markers linked to different polygenic traits at lower costs. By identifying 8 R lines and 1 CMS line pairs as accessions as duplicates, the present study also highlighted the presence of duplicates in R lines maintained at Directorate of Rice Research. However, it is impossible to prove to that the two accessions are genetically identical without sequencing their genomes. From germplasm management point of view, samples do not necessarily have to be completely identical in order to be considered as duplicate/redundant. Quantification of genetic diversity between samples based on screening of germplasm for large number of markers combined with passport data can be used to identify duplicate (Virk *et al*., 1995). It can be concluded that only one of the duplicates can be selected for crossing since, it reduces the efforts for making combinations with CMS lines. The results suggested that a relatively less number of (GATA)n microsatellite markers can be used for the reliable estimation of genetic diversity and identification of duplicates. Though (GATA)n motifs are limited in number, PCR-based markers targeting these motifs are highly polymorphic, as evident from the present study.

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