African Journal of Biotechnology Vol. 11(84), pp. 14956-14995, 18 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.215
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Evaluation of genetic diversity in rice using simple sequence repeats (SSR) markers

Ravindra Kumar*, Ashwani Kumar Singh, Arun Kumar and Radha

Department of Finger Printing, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P -250110, India.

Accepted 5 September, 2012

The genetic diversity of 64 rice genotypes using 20 SSR primers on chromosome number 7-12 was investigated. DNA was extracted by modified cetyl trimethyl ammonium bromide (CTAB) method. The banding pattern was recorded in the form of 0-1 data sheet which was analyzed using unweighted pair group method with arithmetic mean (UPGMA) based on Jaccard's similarity coefficient. The results revealed that out of twenty, eight primers showed distinct polymorphism, indicating the robust nature of microsatellites in revealing polymorphism. The cluster analysis showed higher level of genetic variation among the genotypes. Similarity coefficients ranged from 0.40 to 0.96. The dendrogram revealed 8 major distinct clusters. Higher range of similarity values for related genotypes using simple sequence repeats (SSR) provides greater confidence for the assessment of genetic diversity and relationships. The polymorphism information content (PIC) value for the SSR loci ranged from 0.36 to 0.98. Higher PIC values were associated with higher level of polymorphism. The information obtained from the DNA fingerprinting studies helps to distinctly identify and characterize the various genotypes. Such information can be used in background selections during backcross breeding programs.

Key words: Genetic diversity, simple sequence repeats (SSR) marker, dendrogram.

INTRODUCTION

Rice is the staple food for more than half of the world's population and it is a model plant for genomic research. Rice belongs to the grass family Poaceae, the genus having 21 wild and 2 cultivated species. It has rich genetic diversity in the form of thousands of land races and progenitor species. From the commercial point of view, DNA fingerprinting is a useful tool for varietal protection to prove ownership or derivation of plant lines. Moreover, the analysis of genetic diversity and relationship between or within different species, populations and individuals is a prerequisite towards effective utilization and protection of plant genetic resources (Weising et al., 1995).

As DNA being the only basis of genetic differences between distinct organisms, DNA fingerprinting is presently the ultimate method of biological diversification. In principle, genetic uniqueness is brought about by two factors inheritance and new mutations. Genetic differences between individuals are laid down in the primary sequence of their genomic DNA, the most straight forward method is identifying an individual

*Corresponding author. E-mail: kumarrk2000@yahoo.com.

Abbreviations: CTAB, Cetyl tri-methyl ammonium bromide; **PCR,** polymerase chain reaction; **EDTA,** ethylenediamine tetraacetic acid; **SSRs,** simple sequence repeats; **RFLP,** restriction fragment length polymorphism; **RAPD,** random amplification of polymorphic DNA; **AFLPs,** amplified fragment length polymorphisms.

sequence for genomes under comparison (Krawczak and Schmidtke, 1994). Morphological and biochemical markers may be affected by environmental factors and growth practices (Xiao et al., 1996; Ovesna et al., 2002; Higgins, 1984). However, DNA markers portray genome sequence composition, thus, enabling to detect differences in the genetic information carried by the different individuals. A wide variety of DNA-based markers have been developed in the past few years. Restriction fragment length polymorphism (RFLP) was the first molecular marker (Botstein et al., 1980), generated for genome analysis and mapping. However, the development of the polymerase chain reaction (PCR) technology has introduced a considerable number of useful molecular markers, for example, random amplification of polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990), amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995) and simple sequence repeats (SSRs) (Cregan, 1992). Since each marker system has specific advantages and disadvantages, the choice of the marker system to be used is the most crucial decision. Recently, the efficacies of different classes of PCR based markers were also used to characterize barley and rice varieties (Saker et al., 2005; Virk et al., 2000). Microsatellite DNA markers consisting of AT repeats were found to be highly polymorphic in the rice genome and could distinguish even closely related *Japonica* cultivars in Japan (Akagi et al., 1997). The objective of current studies was to estimate the genetic diversity among sixty four genotypes of diverse genetic background and to develop unique fingerprint for each genotype. These genotypes were selected to test their performance under direct sown aerobic conditions.

MATERIALS AND METHODS

Plant material

A field experiment was conducted during wet season of 2010-2011 at Crop Research Center (CRC) of Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P., India. A total of sixty four selected rice genotypes were used for the genetic variability analysis under aerobic field conditions (Table 1). The selected genotypes were developed at International Rice Research Institute (IRRI), Philippines and different rice improvement centers across India.

DNA extraction

The leaf samples were secured from the above mentioned aerobic rice field experiment. Total DNA was extracted from fresh leaves by the cetyl tri-methyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). The quality and concentration of extracted DNA were estimated by using a UV-Vis spectrophotometer. The DNA was spooled out, washed twice with 70% ethanol and dissolved in TE (10 mM Tris, 0.1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0) containing 25 µg/ml RNase-A, incubated at 37°C for 30 min and extracted with chloroform:*iso*-amyl alcohol (24:1 v/v). DNA was re-precipitated and dissolved in TE buffer. DNA was checked for its quality and quantity by 0.8% agarose gel electrophoresis.

PCR analysis and gel electrophoresis

A set of twenty SSR primers were used. The details of SSR primers used in the investigation are presented in Table 2. The PCR reaction was carried out using Taq polymerase in 20 ml reaction volume containing 1.5X PCR buffer, 2 mM $MgCl_2$, 0.02 mM of each dNTPs, 1 mM of forward and reverse primers, 0.5 µl (3 unit) Taq polymerase and 50 ng genomic DNA. Profile was used as follows: an initial hot start and denaturing step at 95°C for 5 min followed by 35 cycles at 94°C for 1 min, appropriate annealing temperature 55°C for 1 min, and primer elongation at 72°C for 2 min. Final extension step at 72°C for 7 min was performed. The SSR-PCR product was analyzed on 4% agarose gel and was visualized by staining with ethidium bromide and transillumination under short-wave UV light. DNA ladder used in the electrophoresis was 100 bp.

Data analysis

Pair wise comparison of genotypes, based on the presence (1) or absence (0) of unique and shared polymorphic products was used to generate Jaccord's coefficient by NT-SYS-pc version 2.1 software (Rohlf, 2000). The similarity coefficient was used to construct a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) (Rohlf, 1993). Combined analysis was performed by using dendrogram along with Jaccard's coefficient. The polymorphism information content (PIC) value described by Botstein et al. (1980) and modified by Anderson et al. (1993) for self-pollinated species was calculated as follows:

$$\mathrm{PIC}_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Where, p_i equals the frequency of the *i*th allele and p_j the frequency of the allele. Only data from polymorphic loci were used for this analysis. The above mentioned methods are used for estimating the results. Primers, where no result was obtained was shown as (-) symbol.

RESULTS

Number of alleles

Among 20 SSR primers, 8 SSRs were with polymorphic loci which were fully distributed on all the rice genomes that had been prescreened; a total of 467 alleles were detected among the 64 genotypes of rice. The overall size of amplified products ranged from 100 to 250 bp. The number of alleles per locus varied from 1 to 10. The SSR primer pairs used for the genetic variability analysis, the number of polymorphic alleles, monomorphic alleles for each SSR locus and PIC values are as shown in Table 3.

The highest number of alleles were observed in RM320 Table 2 and RM264 (ten alleles each) followed by RM206 (eight alleles), RM47, (six alleles each), RM21,

Table 1. List of selected rice genotypes used in investigation.

Genotype code no.	Genotype name	Sources/origin
1	Govind	GBPUAT, Pantnagar, India
2	CB08-709	TNAU, Coimbatore, India
3	CB-05-758	TNAU, Coimbatore, India
4	CB06-803	TNAU, Coimbatore, India
5	Pant Dhan-6	GBPUAT, Pantnagar, India
6	Pant Dhan-16	GBPUAT, Pantnagar, India
7	CR2703-125-16-1-5-1	CRRI/IRRI, Cuttack, India
8	CR2707-183-1-1-1	CRRI/IRRI, Cuttack, India
9	CR2707-120-32-1-3-1	CRRI/IRRI, Cuttack, India
10	CR2707-185-10-1-4-1	CRRI/IRRI, Cuttack, India
11	CR2701-120-1-1-2-1	CRRI/IRRI, Cuttack, India
12	CR2707-185-23-1-3-1	CRRI/IRRI, Cuttack, India
13	CR2704-127-23-1-1-1	CRRI/IRRI, Cuttack, India
14	CR2703-125-20-1-2-1	CRRI/IRRI, Cuttack, India
15	CR2707-185-16-1-1-1	CRRI/IRRI, Cuttack, India
16	CR2706-171-14-1-4-1	CRRI/IRRI, Cuttack, India
17	CR2707-185-9-1-1-1	CRRI/IRRI, Cuttack, India
18	CR2703-125-22-1-1-1	CRRI/IRRI, Cuttack, India
19	CR2703-125-7-1-3-1	CRRI/IRRI, Cuttack, India
20	CR2698-121-8-1-3-1	CRRI/IRRI, Cuttack, India
21	CR2701-119-49-1-2-1	CRRI/IRRI, Cuttack, India
22	CR2701-120-49-1-2-1	CRRI/IRRI, Cuttack, India
23	CR2698-121-5-1-3-1	CRRI/IRRI, Cuttack, India
24	CR2707-184-2-1-1	CRRI/IRRI, Cuttack, India
25	CR2706-165-4-1-2-1	CRRI/IRRI, Cuttack, India
26	CR2701-118-40A-1-3-1	CRRI/IRRI, Cuttack, India
27	CR2706-165-10-1-4-1	CRRI/IRRI, Cuttack, India
28	CR2704-127-22-1-3-1	CRRI/IRRI, Cuttack, India
29	CR2698-121-22-1-7-1	CRRI/IRRI, Cuttack, India
30	CR2704-127-29-1-2-1	CRRI/IRRI, Cuttack, India
31	CR2704-185-25-1-1-1	CRRI/IRRI, Cuttack, India
32	CR2703-125-11-1-5-1	CRRI/IRRI, Cuttack, India
33	CR2706-165-9-1-2-1	CRRI/IRRI, Cuttack, India
34	IR83871-B-B-237-CRA-5-1-1	IIRI, Philippines
35	IR83931-B-B-305-CRA-2-1-1	IIRI, Philippines
36	IR83870-B-B-231-CRA-5-1-1	IIRI, Philippines
37	IR83927-B-B-278-CRA-1-1-1	IIRI, Philippines
38	IR84887-B-156-CRA-14-1-1-1	IIRI, Philippines
39	IR83927-B-B-278-CRA-5-1-1	IIRI, Philippines
40	IR84887-B-152-CRA-10-1-1	IIRI, Philippines
41	IR84896-159-CRA-25-1-1-1	IIRI, Philippines
42	Rasi	DRR, Hyderabad, India
43	Tulasi	DRR, Hyderabad, India
44	Nidhi	DRR, Hyderabad, India
45	PR115	PAU, Ludhiana, India
46	PR120	PAU, Ludhiana, India
47	Birsa Dhan-201	Birsa Agri.Uni.Bihar, India
48	Punjab Mehak-1	PAU, Ludhiana, India
49	3064	PAU, Ludhiana, India

Table 1. Continued.

50	3065	PAU, Ludhiana, India
51	3068	PAU, Ludhiana, India
52	CB 0015-10	TNAU, Coimbatore, India
53	Pant Dhan-11	GBPUAT, Pantnagar, India
54	Krishna Hasma	DRR, Hyderabad, India
55	Vikas	DRR, Hyderabad, India
56	Sasyasree	DRR, Hyderabad, India
57	Triguna	DRR, Hyderabad, India
58	ARB-6	UAS, Bangalore, India
59	Arix6129	Bayer Bioscience, Ltd, India
60	PAU-201	PAU, Ludhiana, India
61	Sarjoo-52	NDUA&T, Faizabad, India
62	PR-114	PAU, Ludhiana, India
63	NDR-359	NDUA&T, Faizabad, India
64	IR- 2511-18	IIRI, Philippines

Table 2. List of primers and their sequences used for the analysis of different genotypes.

S/N	Primer code	Manufacturer	Primer sequences 5'-3'	
			Forward	Reverse
1	RM 21	Merk (Genei), India	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAC
2	RM 536	-do-	TCTCTCCTCTTGTTTGGCTC	ACACACCAACACGACCACAC
3	RM 206	-do-	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG
4	RM 566	-do-	ACCCAACTACGATCAGCTCG	CTCCAGGAACACGCTCTTTC
5	RM 320	-do-	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC
6	RM 247	-do-	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG
7	RM 544	-do-	TGTGAGCCTGAGCAATAACG	GAAGCGTGTGATATCGCATG
8	RM346	-do-	CGAGAGAGCCCATAACTACG	ACAAGACGACGAGGAGGGAC
9	RM 547	-do-	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATTCTCGTAGCG
10	RM 519	-do-	AGAGAGCCCCTAAATTTCCG	AGGTACGCTCACCTGTGGAC
11	RM286	-do-	GGCTTCATCTTTGGCGAC	CCGGATTCACGAGATAAACTC
12	RM 561	-do-	GAGCTGTTTTGGACTACGGC	GAGTAGCTTTCTCCCACCCC
13	RM 222	-do-	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG
14	RM 47	-do-	ACTCCACTCCACTCCCCAC	GTCAGCAGGTCGGACGTC
15	RM 10	-do-	TTGTCAAGAGGAGGCATCG	CAGAATGGGAAATGGGTCC
16	RM 19	-do-	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA
17	RM 20	-do-	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG
18	RM R	-do-	ACGAGCTCTCGATCAGCCTA	TCGGTCTCCATGTCCCAC
19	RM 167	-do-	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC
20	RM 264	-do-	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATTAGC

RM561 (six alleles each), RM346 (five alleles) and lowest number of alleles were observed in RM247, (four alleles) that provides the summarized data regarding the number of unique alleles and their distribution in various entries. No results (-) were obtained from twelve SSR primers namely RM536, RM566, RM544, RM547, RM519, RM286, RM222, RM10, RM19, RM20, RM167and RMR.

Polymorphism of SSR markers

The alleles revealed by SSR markers showed a high

Table 3. Polymorphism Information Content (PIC) of SSR Loci across various varieties analyzed in the investigation.

S/N	Primer code	Chromosome location	Motifs	Molecular wt. range (bp)	Total no. of alleles	No. of polymorphic alleles	No. of monomorphic alleles	% Polymorphism	Diversity in value of PIC
1	RM 21	11	(GA)18	120-180	55	6	0	100	0.75
2	RM 536	11	(CT)16	-	-	-	-	-	-
3	RM 206	11	(CT)21	200-250	64	8	0	100	0.42
4	RM 566	9	(AG)15	-	-	-	-	-	-
5	RM 320	7	(AT)11GTAT (GT13)GT13)	130-160	40	10	0	100	0.60
6	RM 247	12	(CT)16	140-200	60	4	0	100	0.36
7	RM 544	8	(TC)9	-	-	-	-	-	-
8	RM346	7	(CTT)18	150-180	42	5	0	100	0.60
9	RM 547	8	(ATT)19	-	-	-	-	-	-
10	RM 519	12	(AAG)8	-	-	-	-	-	-
11	RM 286	11	(GA)16	-	-	-	-	-	-
12	RM 561	-		140-220	65	6	0	100	0.70
13	RM 222	10	(CT)18	-	-	-	-	-	-
14	RM 47	7	(AG)7(AG)11	100-220	70	6	0	100	0.98
15	RM 10	7	(GA)15	-	-	-	-	-	-
16	RM 19	12	(ATC)10	-	-	-	-	-	-
17	RM 20	12	(ATT)14	-	-	-	-	-	-
18	RM R	-	-	-	-	-	-	-	-
19	RM 167	11	(GA)16	-	-	-	-	-	-
20	RM 264	8	(GA)27	130-150	71	10	0	100	0.72

degree of polymorphism; with as many as 8 primers produced 100% polymorphic bands. A total of 467 bands were obtained from 8 SSR primers, of which all were polymorphic, with an average of 58.37 bands per primer. The PIC values, derived from allelic diversity and frequency among the genotypes, were not uniform for all of the SSR loci tested. The PIC value for the SSR loci ranged from 0.36 to 0.98. The highest PIC value (0.98) was observed for primer RM47 followed by RM21 (0.75), RM561 (0.70), RM320 and RM346 with 0.60 each and RM206 (0.42). The lowest PIC value (0.36) was observed for the Primer RM247. Primer RM536, RM566, RM544, RM547, RM519, RM286, RM222, RM10, RM19, RM20, RM167, and RMR provided no (-) result.

Similarity versus dissimilarity analysis

The dendrogram revealed 8 clusters among the genotypes. Jaccard coefficient showed a cut-off similarity coefficient level of 0.4, below which the

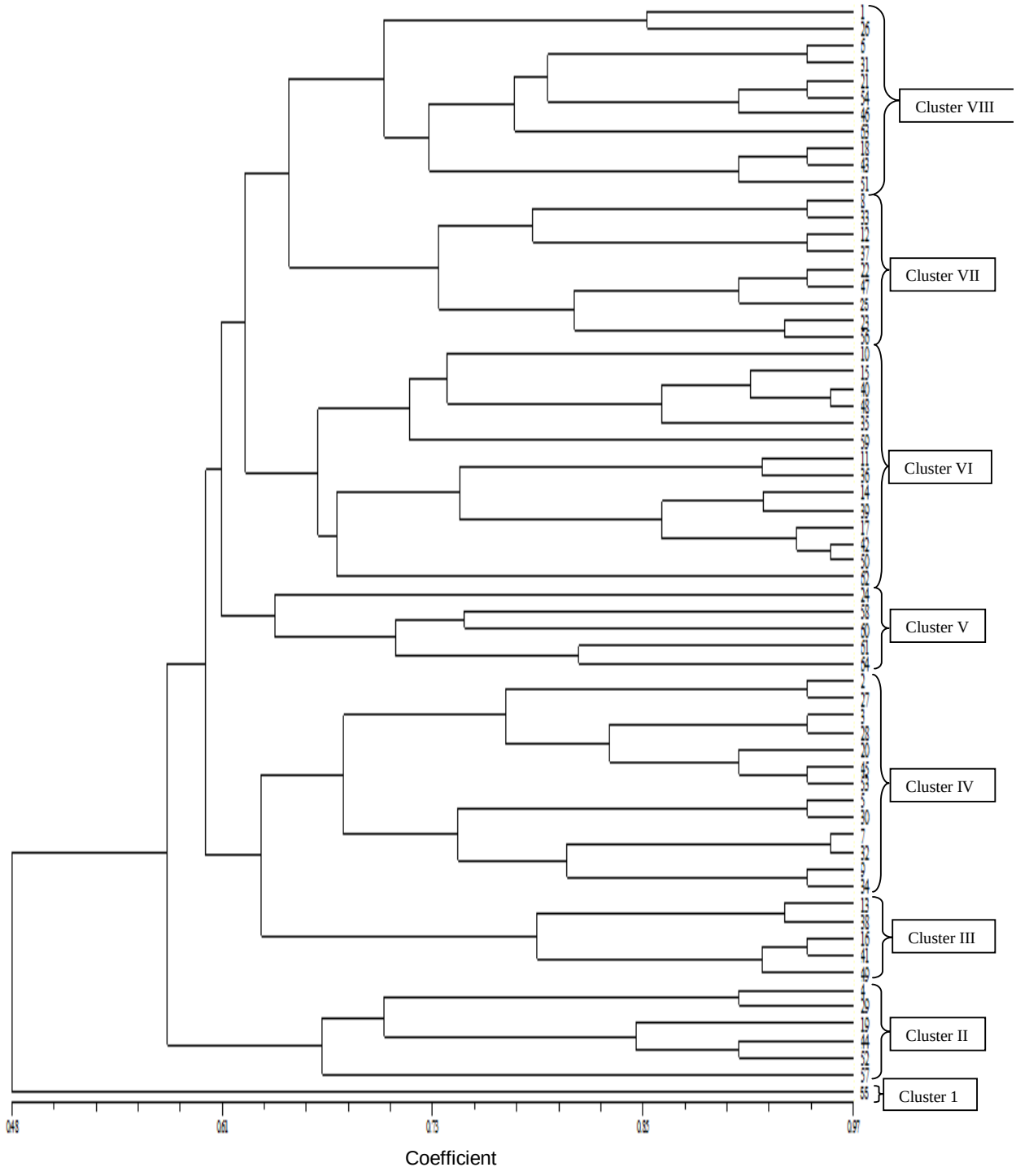

Figure 1. Dendrogram showing clustering of 64 genotypes of rice constructed by using UPGMA cluster analysis of genetic similarity based on SSR data.

similarity values narrowed conspicuously. Cluster VI, IV, VIII, VII, II, III and V comprised of 14, 13, 11, 9, 6, 5 and 5 genotypes, respectively (Figure 1). Jaccard's coefficient of similarity revealed that high degree of similarity (96%) was observed between IR84887-B-152-CRA-10-1-1 and Punjab Mehak, thus, are genetically similar. Whereas

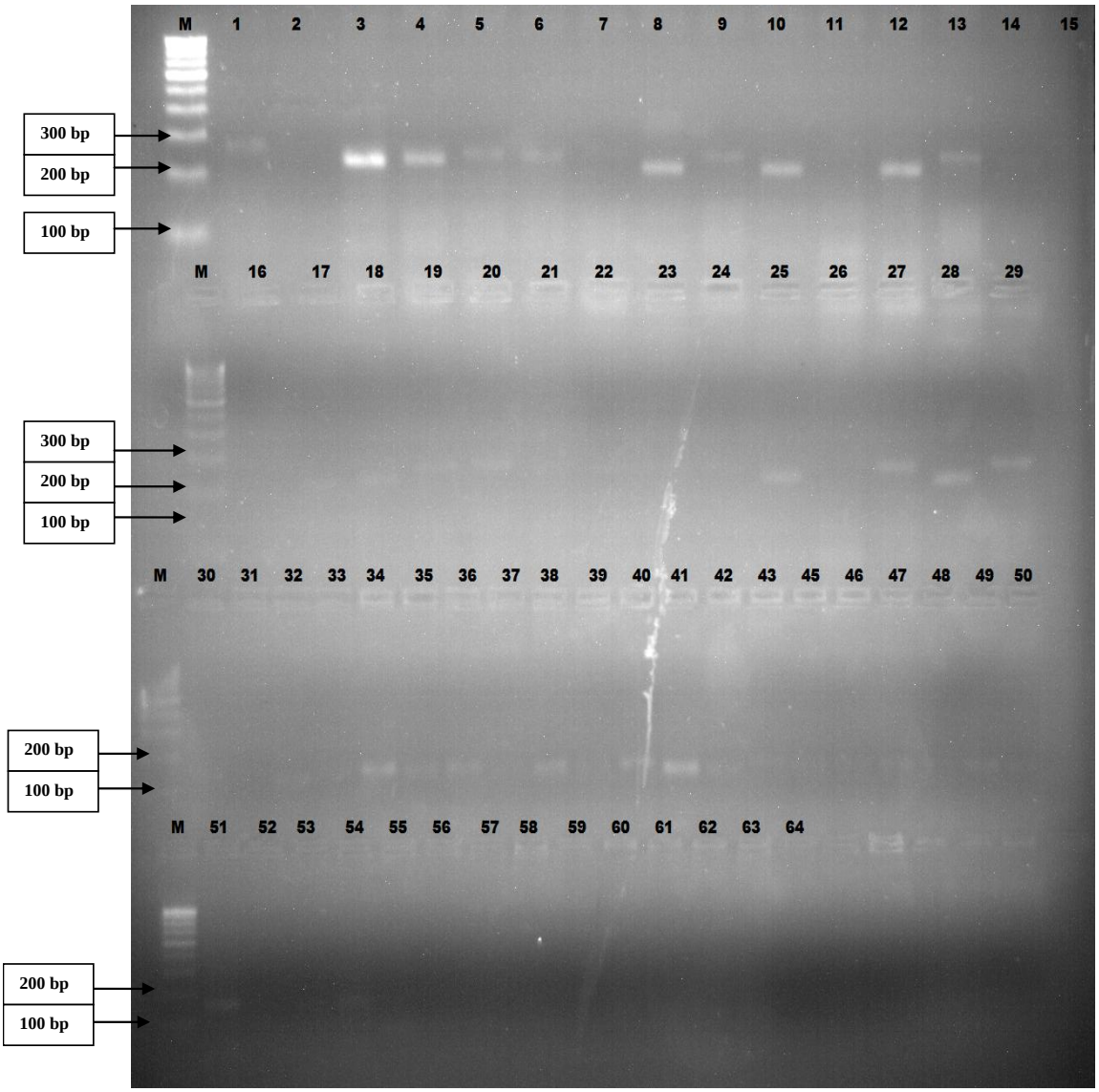

Figure 2. Agarose gel electrophoresis of PCR products of the microsatellite marker RM 320 of 64 rice varieties; M= DNA ladder (100 bp).

CR2706-165-4-1-2-1 keeps low level of similarity range, that is, 34 to 69% to the members of other clusters. The input matrix for genetic analysis was prepared from the scoring of observed main alleles. Cluster analysis showed a significant genetic variation among the genotypes, with similarity coefficient ranging from 0.4 to 0.96. Similarity coefficient ranged between 0.34 and 0.69 in cluster I with other clusters of dendrogram. Cluster II with the similarity coefficient ranged between 0.62 and 0.90. Clusters III, IV, V and VI with the similarity coefficient ranged between 7.3 and 0.94, 0.54 and 0.94, 0.57 and 0.81, and 0.46 and 0.97, respectively. While cluster VII and VIII with similarity coefficient ranged between 0.62 and 0.94.

DISCUSSION

The 20 SSR markers already mapped in rice with higher level of polymorphism as described by Brondani et al. (2001), Rangel (2008) and www. gramene.org, were used for studies (Figure 2). The sequences of primers were obtained from the public website (http: //www. gramene.org/microsat/microsats.txt). SSR markers used in studies were synthesized by Bangalore Genie, Merk India, were distributed from chromosome numbers 7-12. Out of 20 primers, only 8 primers generated banding pattern. The number of alleles per locus varied from 4 to 10. The average number of alleles per locus was 8.49, indicating a greater magnitude of diversity among the

plant materials included in the present investigation. A total of 467 bands were scored of which none was monomorphic. The eight identified markers are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate at a specific locus in rice. Similar results were also reported by DeWoody et al. (1995). SSR primers namely, RM21, RM561, RM47 and RM264, generated higher levels of polymorphism and any two of them can be used to differentiate rice genotypes. Our results were also substantiate the inferences of Aggarwal et al. (2002) that SSR data sets generated from two or three primer combinations are sufficient for robust estimates and that additional data sets do not change the relationships among the rice genotypes. According to Akkaya and Buyukunal-Bal (2004), high PIC value can be attributed to the informative markers. Higher PIC values were observed with SSR primer RM47 (0.98), RM21 (0.75) and RM264 (0.72). This observed pattern was consistent with the findings of Lapitan et al. (2007).

The SSR markers showed an average PIC value of 0.64. Markers with PIC values of 0.5 or higher are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at a specific locus (DeWoody et al., 1995).

The mean PIC value observed in current studies was higher than the PIC value of 0.56 as recorded by Ravi et al. (2003) with rice cultivars, landraces and wild relatives. This indicated that the genotypes used in the present study were diverse due to differences in origin, ecotype and speciation. The dendrogram (Figure 1) resulting from UPGMA analysis revealed allelic richness of 8 clusters for various sizes. Based on the present study, the large range of similarity values for related genotypes using microsatellites provide greater confidence for the assessment of genetic diversity and relationships. Such fingerprinting makes identification and characterization of genotypes very easy and will be helpful in background selections during back cross breeding programme.

Varietal profiling based on SSR markers will be more reliable as compared to other markers, since SSR markers detect finer levels of variations among closely related lines.

ACKNOWLEDGEMENTS

Current investigation was funded by National Agriculture Development Project, New Delhi, India. We are also grateful for the help of Dr. Mahesh Gathala Associate Scientist, IRRI, Philippines for providing the seed material for field experiment.

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