Molecular Characterization of Rice Germplasm (*Oryza sativa* L.) using Simple Sequence Repeat (SSR) Markers

M. Okello[1], S. V. Sawardekar[1*], N. B. Ghokhale[1], B. D. Waghmode[2] and D. M. Patil[1]
[1] Plant Biotechnology Centre, College of Agriculture, Dapoli
[2] Regional Research Station, Shirgaon, Ratnagiri
Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri - 415 712 (MS)

Abstract

The present study was carried out to screen forty eight rice germplasm with 18 simple sequence repeat (SSR) markers to study their genetic diversity and phylogeny structure. Each primer showed 100% polymorphism. A total of 275 alleles were generated by 18 primers and each primer produced on an average 15.27 alleles of the size ranging from 172.22 bp (base pair) to 329.44 bp. The number of alleles amplified for each primer pair was ranged from 5 to 35. The markers pTA-248 generated a maximum number of alleles (35), while the primer RM-309 produced minimum number of alleles (5). The polymorphism information content (PIC) values of primers ranged from 0.58 (RM-206) to 0.85 (RM-140) with an average PIC value of 0.77. It was also observed that there was no correlation between percentage polymorphism and PIC value as SSR primer RM-206 showed minimum PIC value but were 100% polymorphic. The higher the PIC value, the more informative is the primer, thus, primers RM-140 and RM-122 were found to be more informative. All the 48 rice genotypes were separated into two major cluster patterns using Jaccard'ssimil aritycoefficientmatrices.

Keywords: Molecular diversity, SSR markers, rice germplasm, polymorphism.

Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world population and is one of the most important food crops grown worldwide (Sasaki and Burr 2000). Almost 11% of the earth's cultivated land area over a wide number of ecosystems is occupied with rice (Cuevas and Fitzgerald, 2012). Currently, there are more than 40,000 rice varieties reported worldwide. Not much of these germplasm has been utilized in the breeding programmes. Hastening of breeding programmes depend upon the number of germplasm available in the population carrying unique heritable resistance traits. The assessment of phenotype may not be a reliable measure of genetic differences since it will change according to the environmental condition. The rapid development of biotechnology allows easy analysis of a large number of loci distributed throughout the genome of plants. Molecular markers can have a number of applications in agriculture, and their application in rice improvement has been reviewed (Collard and Mackill 2008; Jena and Mackill 2008).

Molecular markers were proven to be powerful tool in the assessment of genetic variation and elucidation of genetic relationships within and among species (Matin *et al.* 2012). SSR markers are useful tool for estimation of genetic distances and identification of germplasm. These markers have some merits like quickness, simplicity, rich polymorphism and stability, thus being widely used in molecular map construction and gene mapping, construction of fingerprints and genetic purity test (Ma *et al.* 2011), analysis of germplasm diversity (Jin *et al.* 2010), utilization of heterosis, especially in identification of species with closer genetic relationship. SSR markers are more popular in rice because they are highly informative, mostly monolocus, co dominant, easily analyzed and cost effective (Prabakaran *et al.* 2010). The basic objective of varietal characterization is to test the occurrence of traits that helps in identifying a particular variety. The characters that are used to distinguish cultivars should have the ability of precise

***Corresponding author :** svsawardekar@rediffmail.com

description and recognition and is considered important only when they are not subjected to environmental influences. Thus, the ability to identify and distinguish between varieties is a fundamental component in rice seed quality programmes. This also benefits the seed production and certification authorities as well as the farmers in ensuring supply and distribution of genetically pure seeds. Using PCR rapid amplification and gel electrophoresis of high resolving power, we can test SSR length polymorphism rapidly and economically. In the current studies, SSR markers and aggregated analysis were carried out to evaluate the genetic diversity among 48 rice germplasm.

Material and Methods

Plant material : Rice germplasm obtained from Agricultural Research Station, Shirgaon district Ratnagiri, Maharashtra, India, were utilized in this study. These germplasm possess biotic and abiotic stress resistance traits. List of germplasm with details are presented in table 1.

DNA extraction: Genomic DNA was isolated from 15 day-old seedlings germinated in potting bags filled with mixture of soil and coco peat following the protocol of Doyle and Doyle (1990) with some modifications of buffer composition and concentration. Purification of DNA in some samples was carried out to remove RNA, proteins and polysaccharides which were the major contaminants. RNA was removed by RNase treatment.

Table 1 : Details of Rice Germplasm used in the study.

Sr. No	Code No.	Name of Variety	Sr. No	Code No.	Name of Variety
1	RTNGM-I-1	Khara Munga	25	RTNGM-I-25	Turga Bhat
2	RTNGM-I-2	Sonphala	26	RTNGM-I-26	Foxtail
3	RTNGM-I-3	SR 3-9	27	RTNGM-I-27	Waksal-2017
4	RTNGM-I-4	Velchi	28	RTNGM-I-28	Jyoti
5	RTNGM-I-5	Manohar Sali	29	RTNGM-I-29	Sorty (Red Kernel)
6	RTNGM-I-6	Agni	30	RTNGM-I-30	Kala Rata
7	RTNGM-I-7	Chinoor	31	RTNGM-I-31	Ralak
8	RTNGM-I-8	Munga	32	RTNGM-I-32	Surak
9	RTNGM-I-9	Panvel-61	33	RTNGM-I-33	RTN Purpal
10	RTNGM-I-10	Norgual	34	RTNGM-I-34	MO-9
11	RTNGM-I-11	Kamod-253	35	RTNGM-I-35	kolhapur Sunil
12	RTNGM-I-12	Narangan	36	RTNGM-I-36	Munga
13	RTNGM-I-13	CO-47-45-120	37	RTNGM-I-37	Bhura Rata
14	RTNGM-I-14	Bela	38	RTNGM-I-38	MO-5
15	RTNGM-I-15	RS-113 (Original)	39	RTNGM-I-39	MO-13
16	RTNGM-I-16	Dular	40	RTNGM-I-40	Rajkamal
17	RTNGM-I-17	MO-8	41	RTNGM-I-41	Pak Basmati
18	RTNGM-I-18	MO-19	42	RTNGM-I-42	Patni-6
19	RTNGM-I-19	Kairli-PTB-49	43	RTNGM-I-43	Bhadas-79
20	RTNGM-I-20	Mayekar Bhat	44	RTNGM-I-44	Bamil
21	RTNGM-I-21	Munga	45	RTNGM-I-45	Pandy
22	RTNGM-I-22	RP Bio 197	46	RTNGM-I-46	Khara Munga
23	RTNGM-I-23	MO-6	47	RTNGM-I-47	Kothambir
24	RTNGM-I-24	MO-17	48	RTNGM-I-48	RP Bio-170

RNase was added to the DNA sample @100 µg ml^{-1} and incubated at 37°C for 1 hour. Concentration of DNA in the sample was determined after agarose gel electrophoresis with standard DNA i.e. uncut Lamda HIND III DNA on 1% agarose gel and by comparison of the intensity of staining with Ethidium Bromide.

Microsatellite assay: Eighteen Simple Sequence Repeat (SSR) markers distributed on all the 12 chromosomes of rice were used for molecular diversity analysis (Table 2). These SSR markers were chosen based on their physical position on the 12 chromosomes of rice genome according to the 'Gramene' database (http//: www.gramene.org). PCR was performed in 96 well plates containing a total 20 µl volume; 1 µl of DNA template, 2.5 µl 10X PCR buffer (containing 200 mM Tris-HCl pH 8.3, 500 mM KCl) 0.5 µl 15 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 0.5 µl of 5 Pmol forward and reverse primers, 0.5 µl of commercial 3U Taq polymerase enzyme (Merck) and 12.5 µl nano-pure water. PCR was run in master thermal cycler and with the following cycle profile; initial denaturation for 5 min at 94ºC and then 30 cycles of 20 sec denaturation at 94ºC, 30 sec annealing at 55°C and 1 min extension at 72ºC and 7 min at 72ºC for the final product extension. PCR amplified products were separated by 2.5% Agarose gel electrophoresis at 80V for 2 to 2.5 h in 1X TAE buffer. Gels were stained in Ethidium bromide solution and visualized under UV light using the gel documentation system (Uvitec Fire reader, UK).

Data analysis: Only clear and unambiguous bands of SSR markers were scored. The sizes of the amplified fragments were estimated with the help of Uvitec fire reader software by Gel documentation system using 100 bp DNA ladder as standard size. Markers were scored for the presence (1) or absence (0) of the corresponding band among the genotypes. UPGMA cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from SSR markers to generate a dendrogram across 48 rice varieties. A pairwise similarity index (SI) was calculated and the UPGMA based dendrogram of 48 rice varieties generated with Multivariate Statistical Package (MVSP). To measure the informativeness of the

Table 2 : SSR markers and their sequences for the primers used in the study.

Sr. No.	Primer	Sequence Forward primer	Sequence Reverse Primer	Chro-mosome No.
1.	RM 140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	1
2.	RM 1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG	1
3.	RM 562	GGAAAGGAAGAATCAGACACAGAGC	GTACCGTTCCTTTCGTCACTTCC	1
4.	RM-8094	AAG TTT GTA CAC ATC GTA TACA	CGCGACCAGTACTACTACTA	1
5.	RM 6775	AATTGATGCAGGTTCAGCAAGC	GGAAATGTGGTTGAGAGTTGAGAGC	6
6.	RM 309	CACGCACCTTTCTGGCTTTCAGC	AGCAACCTCCGACGGGAGAAGG	12
7.	RM 5479	CTCACCATAGCAATCTCCTGTGC	ACTTCGTTCACTTGCATCATGG	12
8.	RM-5926	ATA TAC TGT AGG TCC ATC CA	AGA TAG TAT AGC GTA GCA GC	11
9.	RM 8225	GCGTGTTCAGAAATTAGGATACGG	GATCTCGCCACGTAATTGTTGC	6
10.	RM 206	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	11
11.	RM 212	AAGGTCAAGGAAACAGGGACTGG	AGCCACGAATTCCACTTTCAGC	1
12.	RM 302	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	1
13.	RM 3825	CCACTAGCAGATGATCACAGACG	GAGCACCTCATAAGGGTTTCAGC	1
14.	RM 1233	ATGGGCACGTGTAATTCATTCG	ATCCTCGAAAGTAGGAGTAGGAAAGC	11
15.	pTA248	AGACGCGGAAGGGTGGTTCCCGGA	AGA CCG GGT AAT CGA AAG ATG AAA	11
16.	RM 122	CTTCTTCCGCTTCCTCCCTTCC	TGTACCAGTGCACCGAGAGTTGG	5
17.	RM 22709	CGCGTGGGCGAGACTAATCG	CCTTGACTCCGAGGATTCATTGTCC	8
18.	RM 547	TTGTCAAGATCATCCTCGTAGC	GTCATTCTGCAACCTGAGATCC	8

markers, polymorphism information content (PIC) for each of the SSR markers was computed according to the formula: PIC = 1ΣPi 2- ΣΣPi2 Pj 2 where 'i' is the total number of alleles detected for SSR marker and 'Pi' is the frequency of the i^{th} allele in the set of hundred genotypes investigated and j = i+1.

Results and Discussion

Molecular markers are powerful tools in the assessment of genetic variation and lucidation of genetic relationships within and among the species. Possible application of DNA profiling techniques for plant variety registration and plant breeders rights (DUS i.e. distinctness, uniformity and stability testing) is being studied worldwide (Lin *et al.* 1996). The genotypes under study were,therefore, subjected to molecular markers analysis for characterization. The major advantage of the molecular markers over the conventional markers lies in their ability to cover the whole genome (Helentjaris *et al.* 1985) and this being able to distinguish even closely related varieties which are otherwise non-distinguishable. Moreover, the stability of these markers over different environments, no stage specificity and advent of rapid and workable techniques like microsatellites. Molecular technique is convenient for characterization, also for testing distinctness of varieties and also for future protection (Law *et al.* 1998).

The SSR allele segregation data were used to construct dissimilarity matrix between genotypes using simple matching coefficient (Sokal and Michener 1958). The polymorphism percentage for individual primer was calculated by the ratio of number of polymorphic bands obtained over the total number of bands produced across the 48 rice genotypes. Atotalof 18 SSRprimerpairs,distr ibutedacrossthegenome,wereusedfor molecular analysis of 48ricevarieties, all the 18 microsatellite markers were found to be polymorphic (Figures 1 and 2). The overall average size of amplified products ranged from 172.22

Table 3 : Amplification details of SSR primers.

Sr. No.	Primer	No. of Alleles	Range of Amplification (bp)	PIC
1	RM-8225	8	210-280	0.79
2	RM-206	9	360-450	0.58
3	RM-1233	20	140-340	0.79
4	RM-140	32	130-450	0.85
5	RM-309	5	150-200	0.65
6	RM-1287	16	80-240	0.81
7	RM-6775	15	60-210	0.81
8	RM-302	15	180-330	0.79
9	pTA-248	35	580-930	0.81
10	RM-547	17	180-350	0.77
11	RM-8094	11	130-240	0.79
12	RM-212	19	130-320	0.85
13	RM-122	14	210-350	0.79
14	RM-5926	10	100-200	0.81
15	RM-3825	13	140-270	0.77
16	RM-5479	17	230-400	0.77
17	RM-22709	11	90-200	0.75
18	RM-562	8	90-170	0.71
Total		275	-	-
Average		15.27	172.22-329.44	0.77

bp to 329.44 bp. A total of 275 alleles were obtained using 18 SSR primer pairs with an average of 15.27 alleles per primer (Table 3). The number of alleles amplified for each primer pair ranged from 5 to 35. The markers pTA-248 generated a maximum number of alleles (35). While the primer RM-309 produced minimum number of alleles (5).

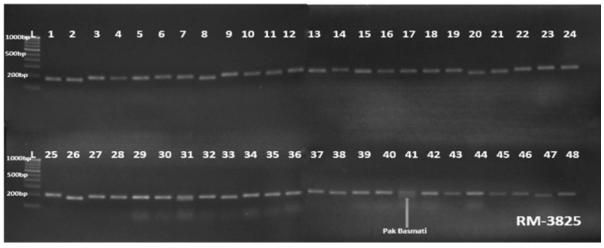

Fig. 1 : SSR PCR assay of marker RM-3825 across 48 rice germplasm in 2.5 % agarose gel. L=100 bp ladder, 1-48: rice germplasm.

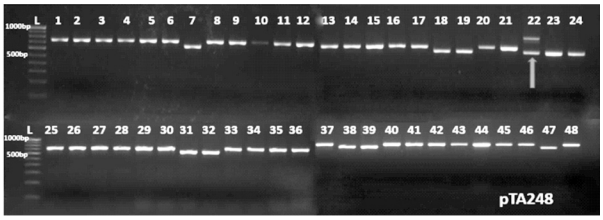

Fig. 2 : SSR PCR assay of marker pTA-248 across 48 rice germplasm in 2.5 % agarose gel. L: 100 bp ladder, 1-48: rice germplasm.

For SSR markers, polymorphism information content (PIC), was calculated as the measure of informativeness of markers (Botstein *et al.* 1980). The PIC values of primers ranged from 0.58 in SSR primer RM-206 to 0.85 in SSR primers RM-140 and RM-122 with an average PIC value of 0.77. Further it was observed that there was no correlation between percentage polymorphism and PIC value as SSR primer RM-206 showed minimum PIC value but were 100% polymorphic. The higher the PIC value, the more informative is the primer, thus, primers RM-140 and RM-122 were found to be more informative.

The diversity observed in the forty-eight rice varieties mainly attributed to the genetic dissimilarities. Pair-wise similarity values ranged from 0 to 0.68. Maximum similarity value of 0.68 was noticed between Manohar Sali and Velchi. Minimum similarity value of 0 was observed between Norgual-Khara Munga, Kamod-253-(Khara Munga, Chinoor), Narangan-(Sonphala, SR 3-9, Velchi, Manohar Sali), Bela-Norgual, RS-113 (origina)-(Chinoor-Kamod-253, Narangan), MO-6-MO-8,MO-17-MO-8, Waksal-2017-Munga, Kala Rata-RP Bio 197, Foxtail-MO-6, Surak-MO-8, MO-5-(RTN Purpal, Munga), Pak Basmati-Munga, Patni-9-Munga, Bhadas-79-Munga, and Bamil-(MO-9, Munga).

Dendrogram was constructed by using Jaccard's similarity coefficient across all the 48 rice germplasm (Fig. 3). Two major clusters were identified (Table 4). The first major cluster consists of two sub clusters. Twelve rice varieties; Kothambir, Khara Munga, Pandy, Bamil, Bhadas-79, Patni-6, RP Bio-170, Pak Basmati, Rajkamal, MO-13, MO-5, Bhura Rata formed the independent first sub cluster. Second sub cluster also consists of twelve varieties *viz.,* Munga, RTN Purpal, Surak, Jyoti, Ralak, Kala Rata, Sorty (Red Kernel), Waksal-2017, Kolhapur Sunil, MO-9, Foxtail, Turga Bhat. The second major cluster consists of two sub clusters. First sub cluster consists of eight rice varieties *viz.,* MO-17, MO-6, RP Bio-197, Narangan, Kamod-253, Munga, Mayekar Bhat, and Norgual. Second sub cluster consist of sixteen rice varieties *viz.,* MO-8, Dular, RS-113 (Original), Kairli-PTB-49, Bela, MO-19, CO-47-45-120, Panvel-61, Munga, Chinoor, Agni, Manohar Sali, Velchi, SR 3-9, Sonphala, and Khara Munga.

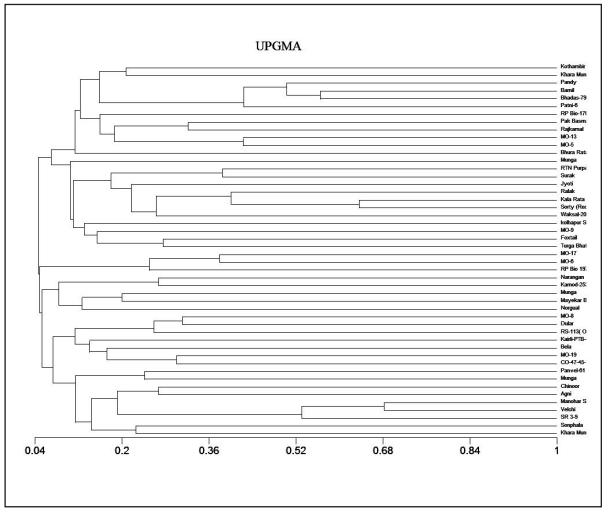

Fig. 3 : Cluster analysis of the 48 rice genotypes using distant coefficient.

Table 4 : Clustering pattern observed for 48 rice varieties.

Cluster		No. of varieties	Name of the varieties
I	IA	12	Kothambir, Khara Munga, Pandy, Bamil, Bhadas-79, Patni-6, RP Bio-170, Pak Basmati, Rajkamal, MO-13, MO-5, Bhura Rata
	IB	12	Munga, RTN Purpal, Surak, Jyoti, Ralak, Kala Rata, Sorty (Red Kernel), Waksal-2017, Kolhapur Sunil, MO-9, Foxtail, Turga Bhat
II	IIA	8	MO-17, MO-6, RP Bio-197, Narangan, Kamod-253, Munga, Mayekar Bhat, Norgual
	IIB	16	MO-8, Dular, RS-113 (Original), Kairli-PTB-49, Bela, MO-19, CO-47-45-120, Panvel-61, Munga, Chinoor, Agni, Manohar Sali, Velchi, SR 3-9, Sonphala, Khara Munga

It is, therefore, of great importance to characterize rice genotypes using DNA- based markers which are cost-effective and give reliable information. Climate change and global warming would be a great challenge to future production of rice. In order to cope with these challenges effectively and increase production for the growing population, we will need to utilize molecular markers especially SSR markers with high selection accuracy for plant diversity sources of which we can then use marker-aided selection for stable resistant gene. Illustrated in this study, SSR primers showed 100% polymorphism which represents the capability of these primers to amplify the less conserved region of the DNA. It also depicted that rice germplasm used in the present screening were, divergent which is a very important factor in breeding programmes.

Acknowledgements

We acknowledge the support of ICAR, Agricultural Research Station, Shirgaon of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. District, Ratnagiri, Maharashtra and also recognize the support of India-African Union fellowship.

References

Collard B C Y and Mackill D J. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society B: Biological Sciences. 363: 557-572.

Cuevas R P, Fitzgerald M A. 2012.Genetic Diversity of Rice Grain QualityGrain Qualityand Nutrition Centre. International Rice Research Institute, Philippines.15:285-310.

Doyle J J and Doyle J L. 1990. Isolation of plant DNA from fresh tissue. Focus. 12: 13-15.

Helentjaris T G K, Solacum M, Siedenstrang C, and Wegman S. 1985. Restriction fragment length polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Molecular Biology. 5: 109- 118.

Jena K and D Mackill. 2008. Molecular markers and their use in marker-assisted selection in rice. Crop Science. 48: 1266-1276.

Jin L, Lu Y, Xiao P, Sun M, Corke H and Bao J. 2010. Genetic diversity and population structure of a diverse set of rice germplasm for association mapping. TAG Theoretical and Applied Genetics. 121: 475-487.

Law J R, Donini P, Koebner M D, Reeves J C and Cooke R J. 1998. DNA profiling and plant variety registration 3. The statistical assessment of distinctness in wheat using amplified fragment length polymorphism. Euphytica. 100: 1-8.

Lin X H, Zhang D P, Xie Y F, Gao H P and Zhang Q. 1996. Identification and mapping of a new gene for bacterial blight resistance in rice based on RFLP markers. Phytopathology. 86: 1156-1159.

Ma H, Yin Y, Guo Z F, Cheng L J, Zhang L, Zhong M and Shao G J. 2011. Establishment of DNA fingerprinting of Liaojing series of japonica rice. Middle-East Journal of Scientific Research . 8(2): 384-392

Matin S, Mohmmad A, Islam M M, Sikdar S U and Zobayer N. 2012. Molecular marker based (SSR) genetic diversity analysis in deep water rice germplasms of Bangladesh. International Journal of Bioscience. 10(2):64-72

Prabakaran A, Paramasivam K, Rajesh T and Rajarajan D. 2010. Molecular characterization of rice land races using SSR markers. Electronic Journal of Plant Breeding. 1(4):512-516.

Sasaki T and Burr B. 2000. International rice genome sequencing project: the effort to completely sequence the rice genome. Current Opinion in Plant Biology. 3: 138–141.

Sokal R R and Michener C D. 1958. A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin. 38:1409–1438.