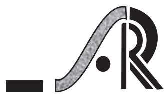

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

Genetic Diversity Studies using SSR and EST-SSR Markers in Maintainer Lines of Rice Hybrids

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Abstract

The performance and heterosis of hybrids are associated with genetic divergence between their parental lines. The present study has been conducted at ICAR-Indian Institute of Rice Research to assess the genetic diversity of 29 rice genotypes including rice hybrid maintainer lines using selected nine EST (Expressed Sequence Tag) based and thirteen reported SSR (Simple Sequence Repeats) markers to identify distinguishable alleles. All nine EST derived microsatellite markers were found to be polymorphic and generated total of 29 alleles in the 29 genotypes. The number of alleles per SSR marker ranged from 2-4 with an average value of 3.2. The polymorphic information content (PIC) for these SSR markers ranged from 0.68 (RMES 9-2) to 0.89 (RMES 5-2) and marker RMES 3-2, RMES 5-2, RMES 6-1, RMES 9-2 generated maximum four alleles. For reported SSR markers, the number of alleles per marker ranged from 1-3 with an average value of 1.9. The PIC value ranged from 0.59 (RM25645) to 0.89 (RM10290) and markers RM25640, RM25645, RM25672, RM10300, RM25641 generated maximum three alleles. This study highlights that EST derived SSR markers are efficient and suitable for assessing the genetic relatedness of the genotypes and studied maintainer lines exhibited high level of genetic diversity.

Keywords: Diversity, Simple sequence repeats (SSR), Expressed Sequence Tag (EST), Hybrid rice, Maintainer lines

Introduction

Rice is the staple food for most of the South Asian countries and it feeds two-third of the world's population. To meet the needs of the growing population of our country, the forecast demand of rice production for the year 2025 is 140 million tonnes. Due to ever increasing limitations in resources, the enhancement of rice production must come from higher absolute yields, which can be met by the hybrid rice technology. Quantitative genetic theory suggests that high heterosis can be expected in a hybrid if the source populations have (i) a high frequency of genes with partial or complete dominance and/or (ii) maximum differences in gene frequencies of over-dominant loci (Hallauer *et al.,* 1988). Consequently, for an optimum exploitation of heterosis, parents should be derived from genetically divergent germplasm pools, commonly referred to as heterotic groups (Melchinger and Gumber, 1998).

Genetic diversity can be measured by different methods such as pedigree analysis, morphological data and molecular markers. Molecular markers have proven useful for assessment of genetic variation in germplasm collections (Mohammadi and Prasanna, 2003) in order to identify the most diverse ones for the development of hybrids.

At present, genomic SSRs are popularly used in rice due to the availability of more than 20,000 markers. But with the current trend towards the use of functional markers, there is enormous scope for the utilization of EST-SSR markers, these are mined from simple sequence repeats (SSRs). Characterization of tall landraces of rice (*Oryza sativa* L.) using gene-derived simple sequence repeats was reported by Neeraja *et al.,* (2005). Analysis of genetic variability based on these functional targets, provides opportunities to study functional diversity and to identify corresponding genes controlling traits of complex inheritance.

The objective of present study is to assess the genetic diversity of maintainer lines with EST derived SSRs and reported SSR markers.

Materials and Methods

Twenty nine genotypes *viz.,* 26 Back Cross Progeny (BCP) lines and three known maintainer lines (Table 1) were used in this study.

Table 1. Rice genotypes selected for the study

Sl. No.	Genotype	Sl. No.	Genotype
1	BCP 48.2	16	BPC 148
2	BCP 85	17	BPC 23
3	BCP 30.2	18	BCP 12.1
4	BCP 35	19	BCP 64
5	BCP 136	20	BCP 139.1
6	BCP 53	21	BCP 154
7	BCP 36.1	22	BCP 3.1
8	BCP 4	23	BCP 138

9	BCP 58	24	1BCP 150
10	BCP 105	25	BCP 14.2
11	BCP 107	26	BCP 5.1
12	BCP 13.2	27	97B
13	BPC 132.2	28	25B
14	BCP 87.1	29	APMS 6B
15	BCP 147		

Molecular analysis

The leaf samples from selected plant material were collected in to labeled 1.5 ml eppendorf tubes in morning hours from field and stored in -20C refrigerator. DNA Isolation was done according to the Zhang *et al.,* (1995) method. The isolated genomic DNA was quantified at 280nm using Nano drop UV Spectrophotometer. The cereal EST-SSR database at http://www.graingenes.org/cgi-bin/ace/query/graingenes was used to select rice EST-SSRs that are hyper polymorphic (with repeat length of 60 bp or more) which are uniformly distributed across the rice genome. Total of nine EST-SSRs (from seven chromosomes) and 13 SSR markers were selected and list given in Table 2 & 3. PCR primer pairs for the selected EST-SSR markers were synthesized by M/s Integrated DNA technologies, USA. The genomic DNA of 29 rice genotypes isolated as described earlier were subjected to PCR amplification as per the procedure described by Panaud *et al.,* (1996). PCR amplified products were resolved in 4% super fine resolution (SFR) agarose gels for EST primers and 3% gels for SSR markers in 0.5X TBE buffer at 200V for 3.5 hrs using Submarine Horizontal Electrophoresis Unit (CBS Scientific, USA). Before loading, PCR amplified products

Table 2. EST-SSR markers selected for the study

Marker Name	Repeat Motif	Forward Primer (5' TO 3')	Reverse Primer (5' TO 3')
Chromosome 2			
RMES 2-1	AG	ACCAAGGCAACCCATGAAT	ACCTGCGGCTTCTTCTTCTT
RMES 2-2	AG	CACCTCCCAATCTTAACCCA	GGGAAGGTGTTGGAGGTGTA
Chromosome 3			
RMES 3-2	AT	ACGGATTCACTGGGTTCTGT	CACCAGAAAGCATCACCTCA
Chromosome 5			
RMES 5-1	AG	TATGATAGCGCCTTCGGAGT	GAGATTAACGTGCGCTCCTC
RMES 5-2	AG	CTCTTACCCCACCAAGGACA	AAAGCGCGCAAAAGAAAAT
Chromosome 6			
RMES 6-1	AT	CTGCCACCGGTGTAGCTAGT	TGGCCCCATCGTATATGAAC
Chromosome 7			
RMES 7-2	AG	TGGCCCTCATGAGACATACA	TTAAGCAATCAAAGGGGGTG
Chromosome 8			
RMES 8-1	AAG	GGAGGAGGAGGAGGATCTTG	CTTCTCCGACGACGAGTTCT
Chromosome 9			
RMES 9-2	AG	CCACGTTGATAAGCTCATTGC	TGGGCACCGAAAATAAAATC

Table 3. SSR markers selected for the study

Marker Name	Chromosome #	Repeat Motif	Forward Primer (5' to 3')	Reverse Primer (5'to 3')
RM 25626	10	GAA	ATGCTCTCAAGTGTGTCAAGG	AACCTCTGGAGTATGTGTAGTGC
RM 25640	10	-	-	-
RM 25645	10	-	-	-
RM 25636	10	AGA	AGCAACACGGGATGGCTAAATCC	AGGTATCGTCTCGGCGTCTCTCC
RM 25669	10	CA	GCAAGGATCACAAACAAAGAGTGC	GGCACCAATTCTAGGAAGGTATGC
RM 25672	10	-	-	-
RM 10290	1	CT	CATCTCGATCAGTCCACCATGC	AGGATTACCATGGCCTCAAGAGC
RM 10296	1	CGC	AAGAGGACCTGCGCCATGAACG	CATCCCTTTCGCCTTCGACTTCC
RM 10303	1	TGGA	TCACTACTACACCCAGCTCGTTCC	TCTCCCTCCTTCACCTGTCTCC
RM 10287	1	-	-	-
RM 10300	1	AGCT	AAAGACAGAATGCCCAGCGATCC	CCTCCACCCATTGGATGACACC
RM 25641	10	-	-	-
RM 25654	10	CGA	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC

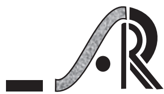

Plate legends:

1	BCP 48.2	7	BCP 36.1	13	BPC 132.2	19	BCP 64	25	BCP 14.2
2	BCP 85	8	BCP 4	14	BCP 87.1	20	BCP 139.1	26	BCP 5.1
3	BCP 30.2	9	BCP 58	15	BCP 147	21	BCP 154	27	97B
4	BCP 35	10	BCP 105	16	BPC 148	22	BCP 3.1	28	25B
5	BCP 136	11	BCP 107	17	BPC 23	23	BCP 138	29	APMS 6B
6	BCP 53	12	BCP 13.2	18	BCP 12.1	24	1BCP 150	30	100 bp ladder

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 100 bp ladder (MBI Fermentas, Lithuania). 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, USA).

Markers were scored for the presence ‘1’ and absence ‘0’ of the corresponding band among the genotypes. A data matrix comprising of ‘1’ and ‘0’ were subjected to cluster analysis. Dendrogram was constructed based on Squared euclidean distance similarity matrix using Jaccard’s coefficient. Data analysis was done using the software NTSYSpc version 2.02 (Rohlf, 1994).

The polymorphism information content (PIC) for each SSR marker was calculated according to the formula:

$$PIC=1-\Sigma Pi^2- \Sigma\Sigma Pi^2Pj^2$$

where ‘i’ is the total number of alleles detected for SSR marker and ‘Pi’ is the frequency of the allele and j=i+1. The PIC value was calculated using the online software-‘Polymorphism Information Content Calculator’ available at *www.agri.huji.ac.*

Result and Discussion

This study was carried out to find genetic diversity of maintainer lines using selected EST derived SSR and reported SSR markers. The extent of genetic diversity in the germplasm can be estimated by adopting various methods like morphological, biochemical and/or molecular analyses. Though number of methods is employed in assessing the genetic diversity of a species, but accuracy of assessment is questionable. The recent developments in molecular biology has resulted in development of simple, easily assayable PCR based DNA markers. Multilocus markers like RAPD, ISSR, AFLP *etc.*, are the most popularly used markers for genetic diversity analysis (Phillips and Vasil, 2001). Among them, SSR markers are useful for a variety of applications in genetics and plant breeding because of their reproducibility, multiallelic nature, co dominant inheritance, relative abundance and good genome coverage.

With the establishment of expressed sequence tag (EST) sequencing projects for gene discovery programs in several plant species, SSR can be identified from these clones and thus generation of EST-SSR markers is relatively easy and inexpensive because they are a byproduct of the sequence data from genes. These are useful as molecular markers because their development is represent transcribed genes, their frequency is abundantly high (Morgante *et al.,* 2002), assumed to reflect more accurately and putative function can often be deduced by a homology search.

In this study, genetic diversity of 29 genotypes was assessed using nine EST-SSR markers and 13 reported SSR markers. EST-SSR markers found polymorphic and generated 29 alleles (Plate 1). The number of alleles per SSR marker ranged from 2-4 with an average value of 3.2. The polymorphic information content (PIC) for these markers ranged from 0.68 (RMES 9-2) to 0.89 (RMES 5-2) and markers RMES 3-2, RMES 5-2, RMES 6-1 and RMES 9-2 generated maximum alleles *i.e.*, four (Table 4). The reported primers were also found polymorphic and generated total of 19 alleles (Plate 2). The number of alleles per SSR marker ranged from 1-3 with an average value of 1.9. The polymorphic information content (PIC) for these 13 SSR markers ranged from 0.59 (RM25645) to 0.89 (RM10290) and markers RM25640, RM25645, RM25672, RM10300 RM25641 generated maximum alleles of three (Table 5). Panaud *et al.,* (1996) and Olufowote *et al.,* (1997) obtained similar number of alleles but higher PIC value (0.89) compared to our study. The PIC values are dependent on the genetic diversity of the accessions chosen. In present investigation, high proportion of closely related cultivars that might be the reason for lower PIC compared to earlier published reports along with EST-SSR primers. It showed less polymorphic compared with genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions (Cho *et al.,* 2000).

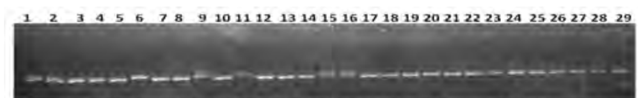

Plate 1: Amplification pattern of the EST-SSR marker RMES 2-2 among the rice lines studied

Plate 1: Amplification pattern of the genomic SSR marker RM25641 among the rice lines studied

Plate legends:

1	BCP48.2	7	BCP36.1	13	BPC132.2	19	BCP64	25	BCP14.2
2	BCP 85	8	BCP4	14	BCP 87.1	20	BCP139.1	26	BCP5.1
3	BCP30.2	9	BCP58	15	BCP147	21	BCP154	27	97B
4	BCP35	10	BCP105	16	BPC148	22	BCP3.1	28	25B
5	BCP136	11	BCP107	17	BPC23	23	BCP138	29	APMS 6B
6	BCP53	12	BCP13.2	18	BCP12.1	24	1BCP150	M	100 bp ladder

Figures:

Table 4. Polymorphism Information of EST-SSR markers

EST marker	Total alleles	Polymorphic Information content (PIC)
RMES 2-1	3	0.876
RMES 2-2	2	0.747
RMES 3-2	4	0.708
RMES 5-1	3	0.817
RMES 5-2	4	0.889
RMES 6-1	4	0.700
RMES 8-1	2	0.840
RMES 7-2	3	0.801
RMES 9-2	4	0.679

Table 5. Polymorphism Information of SSR markers

SSR markers	Total alleles	Polymorphic Information content (PIC)
RM25626	1	0
RM25640	2	0.845422
RM25645	2	0.592747
RM25672	2	0.618906
RM10290	3	0.899326
RM10296	1	0
RM10303	1	0
RM10287	3	0.876338
RM10300	2	0.642687
RM25641	2	0.737218

Cluster analysis was performed using Jaccard's similarity coefficient of 48 alleles and generated dendrogram (Figure 1) with mean genetic similarity of 0.56 (range 0.20 to 1.0) indicating a high diversity. These observations are similar to studies of Garland *et al.,* (1999) who obtained mean genetic similarity of 0.5 (range 0.0 to 1.0) in analysis of genetic diversity of 43 rice cultivars with 10 SSR primer pairs. The dendrogram generated using SSR marker data grouped all the 29 genotypes into two major clusters with 28 percent similarity among them.

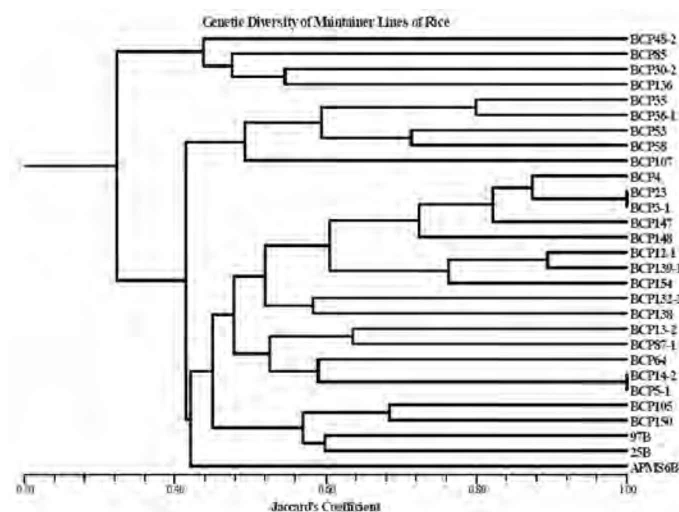

Figure 1. Cluster analysis based on Jacord similarity co-efficient

The use of EST-SSR markers in genetic diversity studies helped in grouping the genotypes according to their genetic relatedness. When more clusters are obtained with few genotypes in each cluster, the significance in clustering is high because of the presence of higher genetic differences between the genotypes in a cluster. Hence, augmented use of EST-SSRs from genes with known functions should be very powerful in unraveling the functional diversity of the genotypes under study.

The present study highlights two important issues *i.e.*, EST-SSR markers are efficient and suitable for assessing the genetic relatedness of the genotypes. Secondly, maintainer lines under study exhibited high level of genetic diversity.

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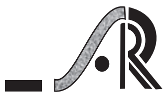

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