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Molecular Characterization of Rice Land Races by SSR Markers

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Abstract

A study was conducted to analysis the molecular characterization of twenty two rice land races at Pandit Jawaharlal Nehru College of Agriculture and Research Institute Karaikal . The SSR marker based diversity analysis grouped the 29 accessions in to 17 clusters which showed its efficiency in grouping the dissimilar individuals. The SSR marker method discriminated the accessions better than the morphological markers. The primer RM 21 identified more number of alleles and based on Polymorphic Information Content (PIC) the primer RM 493 is ascertained as more informative than the rest of the 14 primers used.

Keywords: Rice land races; SSR markers; Clustering; Genetic diversity

Introduction

Land races played an important role in the local food security and sustainable development in agriculture, in addition to their significance as genetic resource for rice genetic improvement [1]. Genetic diversity is conventionally assessed by morphological traits. However, such traits are affected by environment, phenology or development stage of the plant and the type of plant material [2,3]. Further, it requires testing in several replications and environments to establish the genetic contributions, which is highly influenced by several factors. Hence there is a need to go in for a highly reliable and precise method for assessment of genetic variability with no environmental effects. Molecular markers are considered an efficient, powerful tool for the assessment of genetic relationships.

Marker analysis helps to understand the genetic make up of the accessions and also make it possible to analyze the global organization of genetic diversity within a species. In contrast to morphological traits, molecular markers can reveal difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management. Several DNA markers are used for molecular diversity anlaysis of which Simple Sequence Repeats (SSR) or microsatellites are a class of repetitive DNA element, and these are the di-,tri- or tetra nucleotide with 5-50 copies per loci, such as $(AT)_{29}$, $(CAC)_{16}$, or $(GACA)_{32}$. Microsatellites are PCR based markers that are technically efficient, cost effective, and common in rice [4]. Compared to RFLP, microsatellite markers detect a significantly higher degree of polymorphism in rice [5] and are especially suitable for evaluating genetic diversity among closely related rice cultivars (or) accessions [6].

A large number of land races are being cultivated which are well adapted to local ecosystems and withstand the adverse effects such as drought, salinity but with a very poor yield [7]. An increase in the yield potential of these land races and at the same time maintaining the desirable traits would be a major objective in rice breeding programme. However assessment of genetic diversity of rice landraces was not given much thrust and the same needs to be studied to identify genetically diverse accession having desired genes which could be better utilised in crop breeding programme.

Materials and methods

The materials used in the study comprise of twenty nine rice land races collected from Department of Plant Breeding and Genetics, Pandit Jawaharlal Nehru College of Agriculture and Research Institute Karaikal, UT of Puducherry. The details of the genotypes are given in Table 1. A field trial was conducted using twenty nine rice land races (Plate.1). These genotypes were sown in raised nursery bed during Kharif, 2008 and 25 days old seedlings were transplanted to the main field in a Randomized Block Design replicated thrice. Each genotype was transplanted in three rows of 3m length adopting a spacing of 30cm between rows and 20cm between plants. All the recommended agronomic package of practices were adopted during the entire crop growth period.

Genetic diversity studies based on Simple Sequence Repeat (SSR) markers

Isolation of Genomic DNA

All the twenty nine genotypes were sown in laboratory condition for the extraction of DNA from leaf samples. DNA was isolated from five days old seedlings from each of the entries for molecular diversity analysis. DNA was extracted by adopting the protocol developed by Dellaporta et al. [8]

Reagents used Cetyl Trimethyl Ammonium Bromide (CTAB) extraction Buffer (100mL):

CTAB	2% (w/v)
Tris HCl (pH8.0)	200 mM
Sodium Chloride	1.4 M
EDTA (pH 8.0)	20 mM

(Tris, sodium chloride and EDTA were autoclaved and 2% CTAB was added after autoclaving and pre heated before using the buffer)

Liquid Nitrogen

β Mercapta ethanol

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Table 1: Details of genotypes.

Sl.No.	Genotype	Origin
1	Orumanayoor Anakkodan	Kerala
2	Vytilla Anakkodan	Kerala
3	Chettivirippu	Kerala
4	Chettivirippu Kannamalai	Kerala
5	Chithyankottai	Tamil Nadu
6	Chitteani	Kerala
7	Gopalbhag	Tamil Nadu
8	Pokkali	Kerala
9	Jodumani	Tamil Nadu
10	Kadamakudy Pokkali	Kerala
11	Kethanur	Kerala
12	Koorgood	Kerala
13	Kulavazhai	Tamil Nadu
14	Vettaikaraniruppu Kulivedichan	Tamil Nadu
15	Vedaranyam Kulivedichan	Tamil Nadu
16	Njavara	Kerala
17	Pallipuram Pokkali	Kerala
18	Pant Kalanamak 3131	Utttar Pradesh
19	Patnai 23	West Bengal
20	Cherthallai Pokkali	Kerala
21	Edavanakad Pokkali	Kerala
22	Ponnarayan	Kerala
23	Red Thriveni	Kerala
24	Sivappu Chitraikar	Tamil Nadu
25	Sivappu Koompalai	Tamil Nadu
26	Thulasi Manjari	Bihar
27	Vattan	Kerala
28	Vellai Chitraikar	Tamil Nadu
29	Vellaikurikar	Tamil Nadu

Ice-cold Isopropanol

Chloroform: Isoamylalcohol (24:1) (v/v)

Ethanol (70 and 100%)

Extraction of Genomic DNA

One gram of fresh and clean leaves were cut into bits with the help of sterile scissors and transferred to prechilled mortar. The leaf tissues were frozen using liquid nitrogen and ground to fine powder. The fine powder was allowed to thaw in the presence of 800µl of preheated extraction buffer in the polypropylene centrifuge tubes, to this 100µl of β-mercaptaethanol was added and incubated for 30-45 minutes at 65°C in water bath with occasional mixing. The tubes were removed from the water bath, equal volume of 800µl of chloroform: Isoamylalcohol mixture (24:1 v/v) was added and mixed by inversion for 1 hour. It was centrifuged at 10,000 rpm for 20 minutes at room temperature. The clear aqueous phase was transferred to a new sterile tube. Equal volume of Ice-cold isopropanol was added, mixed gently by inversion and then kept in the freezer until DNA was precipitated. The mixture was centrifuged at 13,000 rpm at room temperature for 10 minutes and the supernatant was decanted retaining the pellet. The pellet was washed with 100µl of 70 per cent ethanol and centrifuged at 6000rpm for 5 minutes. The alcohol was discarded and DNA was completely air dried. The dried pellet was resuspended in 100µl of sterile water.

Quantification of DNA

The genomic DNA extracted from the young leaf samples was tested for its intactness by electrophoresis. The DNA was quantified with visually on agarose (0.8%) gel by staining with ethidium bromide. 10µl of each genotype DNA was loaded with tracking dye along with 1 Kb DNA. After the tracking reached one third of the gel, documentation was done using UV transilluminator. The quantity of isolated DNA was determined by its corresponding ladder DNA. Based on this, the dilution of DNA was done for SSR amplification.

PCR Amplification

Sequence of micro satellite primer pairs were downloaded from Genome data bases, Rice genes micro satellite markers (http://www.gramene.org/markers/microsat/ssr.html) and 15 primers were chosen randomly covering all the 12 chromosomes and the details of the primer sequences and the product size are given in Table 2. These primer sequences synthesized by Sigma Aldrich Inc., Bangalore were utilized for amplification.

The total reaction volume was 15µl, the cocktail for the amplification was prepared as follows in 0.2ml PCR tubes.

DNA	2.00µL
dNTPs (2.5mm) (Bangalore Genei Ltd., India)	1.50µL
Primer (Sigma Aldrich Inc., USA)	2.00µL
PCR buffer	1.50µL
Taq Polymerase (3 units/µL) (Bangalore Genei Ltd., India)	0.2µL
Magnesium chloride	0.3µL
Sterile distilled H_2O	7.50µL

Table 2: Details of SSR primers used for PCR amplification.

Sl. No.	Oligo Name	Chromosome location	Sequence (51-31)	Product Size (bp)
1	RM493	1	TAGCTCCAACAGGATCGACC* GTACGTAAACGCGGAAGGTG**	211
2	RM110	2	TCGAAGCCATCCACCAACGAAG* TCCGTACGCCGACGAGGTCGAG**	156
3	RM53	2	GCCTCGAGCATCATCATCAG* ATCAACCTGCACTTGCCTGG**	182
4	RM489	3	ACTTGAGACGATCGGACACC* TCACCCATGGATGTTGTCAG**	271
5	RM261	4	CTACTTCTCCCCTTGTGTCG* TGTACCATCGCCAAATCTCC**	125
6	RM131	4	TCCTCCCTCCCTTCGCCCACTG* CGATGTTCGCCATGGCTGCTCC**	215
7	RM403	5	GCTGTGCATGCAAGTTCATG* ATGGTCCTCATGTTCATGGC**	241
8	RM343	6	CCACGAACCCTTTGCATC* GTGATGATGCGTCGGTTG**	233
9	RM429	7	TCCCTCCAGCAATGTCTTTC* CCTTCATCTTGCTTTCCACC**	159
10	RM481	7	TAGCTAGCCGATTGAATGGC* CTCCACCTCCTATGTTGTTG**	169
11	RM515	8	TAGGACGACCAAAGGGTGAG* TGGCCTGCTCTCTCTCTCTC**	211
12	RM524	9	TGAAGAGCAGGAACCGTAGG* TCTGATATCGGTTCCTTCGG**	198
13	RM222	10	CTTAAATGGGCCACATGCG* CAAAGCTTCCGGCCAAAAG**	213
14	RM21	11	ACAGTATTCCGTAGGCACGG* GCTCCATGAGGGTGGTAGAG**	157
15	RM17	12	TGCCCTGTTATTTTCTTCTCTC* GGTGATCCTTTCCCATTTCA**	184

* Forward primer
**Reverse primer

The reaction mixture was given a short spin for thorough mixing of the cocktail components. Then the 0.2mL PCR-tubes were loaded on to a thermal cycler (MJ Research Inc. USA). The thermal cycler was programmed as follows.

Profile 1:	95°C for 1 minute	Initial denaturation
Profile 2:	94°C for 45 seconds	denaturing
Profile 3:	55°C for 1 minute	Annealing 34 cycles
Profile 4:	72°C for 1 minute 30 seconds	Extension
Profile 5:	72°C for 10 minutes	Final Extension
Profile 6:	4°C for infinity to hold the samples	

Agarose Gel Electrophoresis

After PCR amplification, products were separated by electrophoresis on agarose gels visualized by ethidium bromide staining.

Materials

Loading dye

Bromophenol Blue 0.5% (w/v)

10 x TBE (Tris Borate EDTA) Buffer

Trisbase – 10.76 gm

Boric acid – 5.5 gm

EDTA – 4 ml

(Dissolved in 800mL and made upto 1000mL and stored at 4°C)

Protocol

The open ends of the Pyrex gel casting plate were sealed with cello tape and placed on a perfectly horizontal platform. Agarose (2.5 %) was added to 1X TBE, boiled until the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added. It was then poured in to the gel mould and then comb was placed properly and allowed to solidify. After solidification of the agarose, the comb and the cello tape were removed carefully. The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TBE to a depth of about 1 cm.

Loading the DNA samples

10µl of DNA samples were pipetted on to a parafilm and mixed well with 2µl of loading dye by pipetting up and down for several times. The gel was run at 90 volt until the tracking dye was one third of the gel and bands were visualized and documented in gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA). The viewed picture was photographed and saved for further scrutiny.

Scoring

Clearly resolved, unambiguous bands were scored visually for their presence or absence with each primer. The scores were obtained in the form of matrix with '1' and '0' which indicate the presence and absence of bands in each variety respectively.

Data Analysis

Polymorphic Information Content (PIC) values were calculated for each of the SSR loci using the formula developed by Nei [9]

$PIC = 1 - \sum x^2_k$

Where, x^2_k represents the frequency at the k^{th} allele.

The data of microsatellite markers were analysed using NTSYS-pc statistical package, version 2.02 i [10]. The bands on the gel were scored for each of the SSR primer pairs in each genotype based on the presence or absence of bands generating a matrix of 1 and 0. Informative bands were used to calculate the genetic distance based on Jaccard's similarity coefficient using SIMQUAL procedure. The DNA data of SSR markers for 29 rice land races were clustered using an unweighted pair group method (UPGMA) with the module of SHAN in the NTSYS-pc package.

Genetic Divergence Based On SSR Marker

Polymorphism of SSR markers

A total of 15 microsatellite markers was used to assess the extent of genetic diversity in the 29 rice land races and all of them were found to be polymorphic. As much as 49 alleles were detected among the genotypes. The number of alleles per locus ranged from 2 (RM 110 and RM 343) to 5 (RM21) with an average of 3.3 per locus.(Plate 4 and 5). The PIC values derived from allelic diversity and frequency among the genotypes were not uniform for all the SSR loci tested. The PIC value for 15 primers varied between 0.19 (RM 429 and RM 524) and 0.69 (RM 493) with a mean of 0.38 (Plate 6). In a set of the 15 primers used in this study there were no null alleles. The details on PIC values are given in Table 3.

Genetic diversity pattern

Dendrogram based on UPGMA analysis grouped the 29 genotypes in to different clusters that were demarcated at a similarity coefficient of 0.74. At this level of 74 per cent genetic similarity, the genotypes were grouped into 17 clusters each clusters distinguishes the genotypes clearly from the other. Cluster I had a maximum of ten land races of which five were originated from Kerala (Figure 1). Cluster II has all the two land races which were collected from Kerala. The remaining clusters except cluster VII are menogenotypic. The cluster VII had three land races (Table 4).

Genetic divergence studies based on SSR marker

Characterization and quantification of genetic diversity have been a major goal in evolutionary biology. The analysis of genetic variation within and among the elite breeding materials is of fundamental

Table 3: Allele variation and PIC values for SSR markers identified in 29 genotypes.

Sl.No.	Primer name	Chromosome location	Number of alleles	PIC values
1	RM 493	1	4	0.69
2	RM 110	2	2	0.37
3	RM 53	2	3	0.44
4	RM 489	3	4	0.36
5	RM 261	4	3	0.25
6	RM 131	4	3	0.38
7	RM 403	5	3	0.30
8	RM 343	6	2	0.37
9	RM 429	7	3	0.19
10	RM 481	7	4	0.45
11	RM 515	8	3	0.47
12	RM 524	9	3	0.19
13	RM 222	10	4	0.35
14	RM 21	11	5	0.53
15	RM 17	12	3	0.34

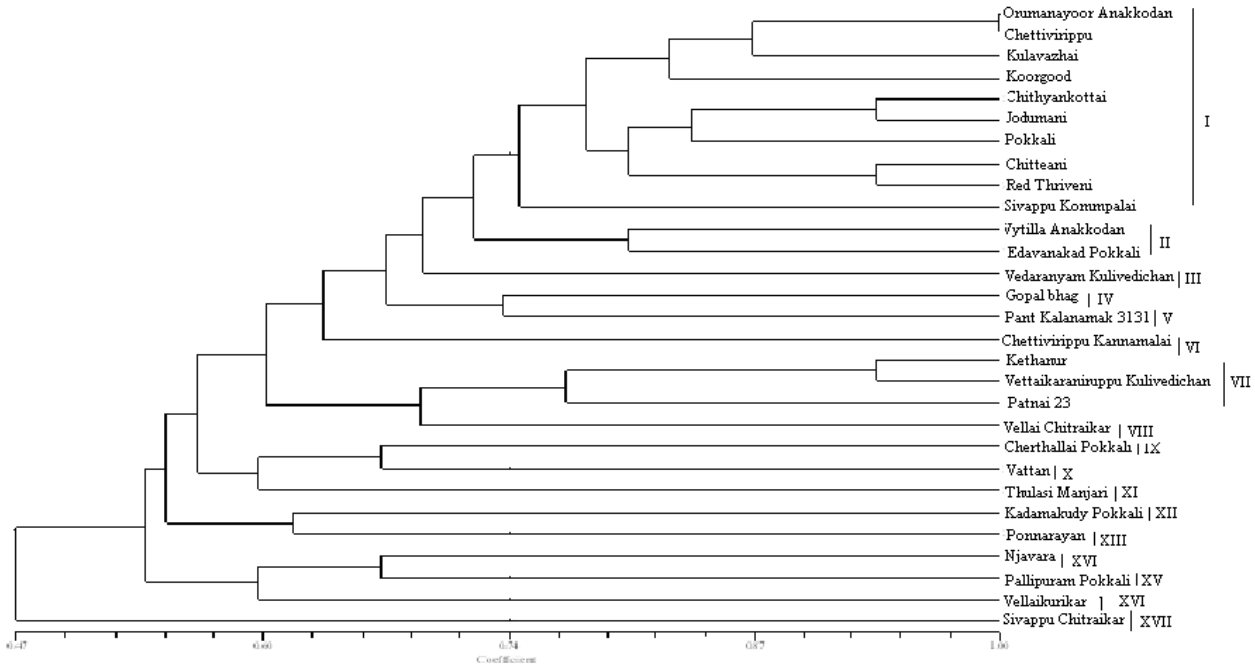

Figure 1: Clustering by UPGMA method for using SSR markers

Table 4: Distribution of genotypes to different clusters based on UPGMA method in genotypic dendrogram.

Cluster No	Number of genotypes	Name of genotypes	Origin
I	10	Orumanayoor Anakkodan	Kerala
		Chettivirippu	Kerala
		Kulavazhai	Tamil Nadu
		Koorgood	Kerala
		Chithyankottai	Tamil Nadu
		Jodumani	Tamil Nadu
		Pokkali	Kerala
		Chitteani	Kerala
		Red Thriveni	Kerala
		Sivappu Koompalai	Tamil Nadu
II	2	Vytilla Anakkodan	Kerala
		Edavanakad Pokkali	Kerala
III	1	Vedaranyam Kulivedichan	Tamil Nadu
IV	1	Gopal bhag	Bangladesh
V	1	Pant Kalanamak 3131	Uttar Pradesh
VI	1	Chettivirippu Kannamalai	Kerala
VII	3	Kethanur	Kerala
		VettaikaraniruppuKulivedichan	Tamil Nadu
		Patnai 23	West Bengal
VIII	1	Vellai Chitraikar	Tamil Nadu
IX	1	Cherthallai Pokkali	Kerala
X	1	Vattan	Kerala
XI	1	Thulasi Manjari	Bihar
XII	1	Kadamakudy Pokkali	Kerala
XIII	1	Ponnarayan	Kerala
XIV	1	Njavara	Kerala
XV	1	Pallipuram Pokkali	Kerala
XVI	1	Vellai kurikar	Tamil Nadu
XVII	1	Sivappu Chitraikar	Tamil Nadu

interest to plant breeders. Diversity based on phenological and morphological characters usually varies with environment and evaluation of these traits requires growing the plant with full maturity prior to identification. To have rapid and easy analysis of a large number of loci distributed throughout the genome of plants, use of molecular marker is increasingly common method and also serves as a powerful tool in the assessment of genetic variation and elucidation of genetic relationships within and among the species. Information regarding genetic variability at molecular level could be used to help, identify and developed genetically unique germplasm that complements existing cultivars.

Cluster analysis of dendrogram constructed using SSR markers

The dendrogram based on UPGMA grouped the 29 genotypes in to 17 clusters that were demarcated at a similarity coefficient of 0.74. Cluster I was the largest and included 10 land races followed by cluster VII and II each of which had three and two land races respectively (Figure 2). The remaining clusters had one genotype each. This dendrogram revealed that the land races that are originated from different places are not grouped together which is indicative of non parallelism exist between geographical origin and genetic diversity.

Polymorphic information content

The term Polymorphic Information Content (PIC) was originally introduced in to human genetics by Botstein et al. [11] and it refers to the value of a marker detecting polymorphism within a population depending on the number of detectable alleles and their distribution of their frequency. In the present investigation, 15 SSR markers revealed 49 alleles in the 29 land races, the number of alleles per locus varied among these markers ranges from 2 to 5 with an average of 3.3 per locus. These kind of less number of alleles per locus was already reported by

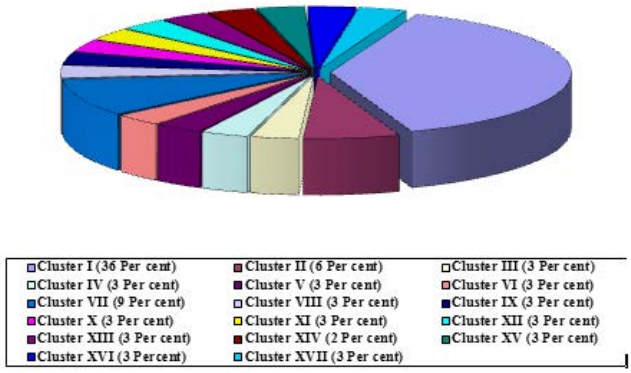

Figure 2: Percentage of genotypes to different clusters based on UPGMA method in Molecular dendrogram.

Ram et al. [12] where an average of 4.86 alleles were detected, indicating less magnitude of diversity among the genotypes. Out of the 15 SSR primers derived from genes, none of them were found monomorphic and however showed average PIC value lesser than 0.50. Lower PIC value may be the result of closely related genotypes and higher PIC values might be the result of diverse genotype. Low PIC values for some other primers were earlier reported by Juneja, et al. [13].

The study also further revealed that the primer RM 21 is ascertained to be showed more number of alleles with PIC value more than 0.50 which indicates the efficiency of this primer in detecting the most heterogenus accession. This is in corabaration with the findings of Giarrocco, et al. [14]. However, the present study indicated that use of more number of markers would be efficient to characterize the land races than used, which highlighted the presence of diversity at genotypic level among the accessions studied.

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