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Molecular Characterization of Aromatic Landraces of Rice (*Oryza sativa* L.) Using Microsatellite Markers

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Abstract

Simple Sequence Repeats (SSR) analysis was performed to assess the genetic diversity in thirty eight aromatic landraces of rice (*Oryza sativa* L.) using 19 SSR primers. The experiment was conducted during *kharif*, 2011 at the Research cum Instructional Farm of Indira Gandhi Krishi Vishwavidyalay, Raipur. 10 SSRs were polymorphic and 3 SSRs were monomorphic while the remaining 6 showed no amplification. The genetic similarity coefficients ranged from 0.40 to 1.00. Cluster analysis was performed using Unweighted Paired Group of Arithmetic Means (UPGMA) using the Jaccard's similarity coefficient. The UPGMA dendrogram resolved the 38 aromatic landraces of rice into two major clusters.

Key words: Aromatic rice, Genetic diversity, Cluster analysis, SSR, Polymorphism

Introduction

Rice (*Oryza sativa* L.) feeds more than 50 % of the tropical populace. A class of aromatic, superfine grade premium rice has evolved its own market niches, making rice trade a commercial success internationally. The Indian aromatic rice, often called Basmati is nature's gift to the sub continent and human kind at large (Ahuja *et al.,* 1995). Basmati rice is highly priced in domestic as well as international markets. Indigenous short aromatic grain (ASG) rice, possess outstanding quality characteristics *viz*., aroma, kernel elongation after cooking (KLAC), fluffiness and taste. However, ASG improvement has been somewhat neglected as they lacked export value. Almost every state of the country has its own set of aromatic rice that performs well in native areas.

Scope of improvement of these landraces depends not only on on the conserved use of genetic variability and diversity but also on the use of new biotechnological tools. Molecular characterization can reveal the maximum genetic variation or genetic relatedness found in a population (Xu *et al*., 2000). Chakravarthi and Naravaneni (2006) reported the usefulness of preservation and conservation of genetic resources since genetic diversity provides information to monitor germplasm and prediction of potential genetic gains. Information regarding genetic variability at molecular level could be used to help, identify and develop genetically unique germplasm that compliments existing cultivars (Ni *et al*., 2002; Ravi *et al*., 2003; Chakravarthi and Naravaneni, 2006). DNA based molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species of rice (Ragunathanchari *et al*., 1999, 2000; Shivapriya and Hittalmani, 2006). The present investigation was undertaken for the assessment of genetic diversity among the aromatic rice landraces with the help of SSR markers.

Materials and Methods

Plant materials and genomic DNA isolation

The plant materials selected for the present study were thirty eight different aromatic landraces of rice (Table 1). The experiment was conducted during the *kharif* season of 2011 at the Research cum Instructional Farm of Indira Gandhi Krishi Vishwavidyalay, Raipur.

Healthy seeds of each variety were sown in pots under appropriate growth conditions for collecting fresh leaves. Total genomic DNA from thirty eight landraces was extracted from six weeks old rice seedlings by mini prep method of DNA extraction. 0.1g of leaf sample was put it into 2 ml eppendorf tube. 0.4 ml of extraction buffer and

beads were added to it and grinded using a crusher. 0.4 ml of 24:1 Chloroform:-Isoamyl alcohol mixture was added and mixed well by vortexing and centrifuged at 14000 rpm for 4 min. Supernatant was collected and transferred to a new eppendorf tube (this action was repeated twice). 0.8 ml of absolute ethanol was added and mixed properly by the tube inversion and centrifuged at 14000 rpm for 4 min. Supernatant was discarded and pellet was washed with 70% Ethanol and air dried for 15-20 min. Pellets were dissolved in 20-40 µl (based on the size of the pellet) of TE buffer or double distilled sterile water and treated with 3 µl RNase for 20 min to remove RNA.

Quantification of DNA

1. Nanodrop spectrophotometer based quantification

Nucleic acid has maximum absorbance of ultra violet light *i.e*., about 260 nm. The ratio between the readings at 260 nm and 280 nm (OD 260/ OD 280) provides an estimate for the purity of nucleic acid. Pure preparation of DNA and RNA has a ratio of approximately 1.8 and 2.0 respectively. If there is contamination with protein or phenol the ratio will be significantly less than this value (< 1.8). A ratio greater than 2.0 indicates a high proportion of RNA in the DNA sample.

2. Dilution of DNA

The crude DNA after quantification was diluted suitably for amplification. DNA was diluted in such a way that the diluted samples contained about 50 ng/µl of crude DNA. Dilution was carried out according to the formula:

$$\text{Dilution} = \frac{\text{Required conc. o f DNA (ng/µl) X Total volume required (µl)}}{\text{Available conc. of crude DNA (ng/µl)}}$$

SSR analysis

For the SSR analysis,2 µl diluted template DNA of each entry was dispensed in PCR plates. Separate cocktail PCR master mix was prepared in an eppendorf tube. The quantity of 18 µl cocktail was added to each well of PCR plates having template DNA. The reaction was carried out in 20 µl reaction volume containing 2 µl (10 ng/ µl) genomic DNA, 11 µl nanopure water, 2 µl 10X PCR buffer, 2 µl 1mM dNTPs, 1 µl Taq DNA polymerase and 2 µl primer (Forward and Reverse). All the reaction chemicals except primers were procured from M/s. Genei, Bangalore, India.

SSR amplification procedure

The PCR tubes were kept in a PCR machine model PTC-100 of MJ research. The DNA was amplified by using profile with some modifications of thermal cycler. Amplification was performed in a thermal cycler with an initial denaturation of 94^0C for 4 min followed by 35 cycles which contains denaturation at 94^0C for 1 min followed by annealing in which the annealing temperature was adjusted based on the Tm value of each primers and finally extension at 72^0C for 5 min.

Electrophoresis and visualization of SSR products

13 µl of PCR amplified SSR was mixed with 2 µl of loading dye (bromophenol) and loaded on 5% polyacrylamide gel prepared in 1X TBE buffer. PBR-322 (ladder) molecular marker was also loaded along with the DNA samples. Electrophoresis was done for 1 hr at 199 volts. The gel along with the DNA samples was then stained with Eithidium bromide (10 µg/10ml) for 40-45 mins. Gel was visualized on UV-transilluminator and images were saved in computer. The banding pattern in the landraces for each set of primers was scored separately. For estimating the size of DNA of each sample, the band position was compared with a base pair of standard marker (PBR-322 ladder). Presence of band in a particular base pair position was scored as "1" and absence of band in a particular base pair position was scored as "0"(zero). The 19 SSR primers used for this purpose are presented in table 2.

Results and Discussion

The results of present study indicated a considerable level of genetic diversity among the cultivars selected. Nineteen SSR markers *viz*., RM-9, RM-215, RM-228, RM-245, RM-247, RM-251, RM-288, RM-302, RM-307, RM-323, RM-335, RM-410, RM-411, RM-433, RM-444, RM-484, RM-506, RM-517 and RM-535 present on different rice chromosomes were studied. There was no amplification with six SSRs (RM 228, RM 245, RM 302, RM 307, RM 323 and RM 517). Ten SSR markers (RM 9, RM 247, RM 251, RM 335, RM 410, RM 411, RM 433, RM 484 RM 444 and RM 535) showed polymorphic reaction, where as three SSR markers RM 215, RM 288 and RM 506 exhibited monomorphic reaction (Fig 1). The dendrogram derived from UPGMA cluster analysis based on similarity coefficient matrix of 38 landraces was constructed. The genetic similarity coefficient for all accessions ranged from 0.40 - 1.00. Results (Fig. 2) indicated that two major groups were formed having 40% similarity *i.e.*

Jawaphool with rest of the aromatic landraces. All the aromatic landraces except Jawaphool were grouped into two classes at nearly 50 % genetic similarity level. Seven genotypes *viz*., Dubraj-1, Dubraj-2, Dubraj-3, Maidubraj, Kharigilas, Kasturi and Dujai clustered together into one group while remaining 30 landraces *viz*., Tulsimanjari, Kalikamod, Atmasheetal, Shuklaphool, Kheraghul, Gangabaru, Jaigundi, Tulsiprasad, Kapoorsar, Chinnor-1, Kubrimohar-1, Jeeradhan, Anterved, Jaophool, Londhi, Samudrafan, Chinnor-2, Kubrimohar-2, Tilkasturi, Elaychi, Lalloo-14, Badhshshbhog, Bisni, Shyamjeera, Katarnibhog, Vishnubhog-2, Gopalbhog, Srikamal, Jeeraphool and Vishnubhog-1 clustered together in the second group.

In group I, Kasturi which is not an aromatic landrace of Chhattisgarh exhibited 55 % similarity with Dubraj group, Dujai and Kharigilas. Kharigilas and rest of the races of group I had 70% genetic similarity. Further, 5 landraces of group I formed two classes which indicated that Dubraj-3 and Maidubraj have genetic similarity nearly 82% according to 13 loci under study.

In second group, Vishnubhog-1 exhibited 61 % genetic similarity with rest of the entries of group II. This group was further divided into two sub groups. With 70 % genetic similarity, group II A included Lalloo-14, Badhshshbhog, Bisni,Shyamjeera,Katarnibhog,Vishnubhog-2,Gopalbhog, Srikamal and Jeeraphool. Whereas group II B included Tulsimanjari, Kalikamod, Atmasheetal, Shuklaphool, Kheraghul, Gangabaru, Jaigundi, Tulsiprasad, Kapoorsar, Chinnor-1, Kubrimohar-1, Jeeradhan, Anterved, Jaophool, Londhi, Samudrafan, Chinnor-2, Kubrimohar-2, Tilkasturi and Elaychi. Kapoorsar, Chinnor-1 and Kubrimohar exhibited 100 % genetic similarity for loci under study. Anterved and Jaophool also exhibited 100 % genetic similarity and also exhibited 91 % genetic similarity with Londhi.Chinnor-2 and Kubrimohar-2 showed similarity for all 13 loci whereas with Tilkasturi, they exhibited 91% genetic similarity. Badhshshbhog, Bisni and Shyamjeera were also found similar for all 13 loci but they were 85% similar with Lalloo-14.

The present investigation reveals that SSR is a valuable tool for estimating the extent of genetic diversity as well as to ascertain the genetic relationship between different cultivars of *Oryza sativa*.

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Table 1. Landraces used in study and places from where they are collected

S. No.	Variety	Source	S. No.	Variety	Source
1	Anterved	Chhuriya/ Rajnandgaon	20.	Londhi	Pendra
2	Atmasheetal	Chhindgarh / Bastar	21.	Mai Dubraj	Bastar
3	Badshahbhog	Jagadalpur	22.	Samudrafan	Pandariya/ Bilaspur
4	Bisni	Surajpur	23.	Shyamjeera	Surajpur
5	Chinnor –I	Balaghat	24.	Srikamal	Raipur Naikin / Sidhi
6	Chinnor-II	Tilda / Raipur	25.	Kalikamod	Aarang/ Raipur
7	Dubraj-I	Nagari	26.	Kapoorsar	Jabera/ Damoh
8	Dubraj-II	Balaodabazar/ Raipur	27.	Kasturi	Bakawand/ Bastar
9	Dubraj-III	Nagri	28.	Kharigilas	Charama/ Bastar
10	Dujai	Pendra	29.	Katarnibhog	Sabour (Bihar)
11	Elaychi	Phingeshwar/ Raipur	30.	Kheraghul	Gharghoda/ Raigarh
12	Gopalbhog	Bagicha, Sarguja	31.	Kubrimohar-I	Bemetra
13	Gangabaru	Chhindgarh/Bastar	32.	Kubrimohar-II	Magarload/ Raipur
14	Jaigundi	Saraypali / Raipur	33.	Sukalaphool	Jaijepur/ Bilaspur
15	Javaphool	Raigarh	34.	Tilkasturi	Pithora/ Raipur
16	Jeeradhan	Tilda / Raipur	35.	Tulasiprasad	Aarang/ Raipur
17	Jeeraphool	Bageecha, Sarguja	36.	Tulsimanjari	Sabour / Bihar
18	Jaophool	Lallunga / Raigarh	37.	Vishnubhog-I	Pendra
19	Lalloo-14	Mandla	38.	Vishnubhog-II	Badrafnagar/ Sarguja

Table 2. Microsatellite markers used for genetic diversification among 38 rice landraces

	SSR Primers	PRIMER SEQUENCES	
		FORWARD 5' → 3'	REVERSE 5' → 3'
1	RM 9	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC
9	RM 215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG
10	RM 228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
9	RM 245	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG
12	RM 247	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG
3	RM 251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
9	RM 288	CCGGTCAGTTCAAGCTCTG	ACGTACGGACGTGACGAC
1	RM 302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC
4	RM 307	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC
1	RM 323	CAACGAGCAAATCAGGTCAG	GTTTTGATCCTAAGGCTGCTG
4	RM 335	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG
9	RM 410	GCTCAACGTTTCGTTCCTG	GAAGATGCGTAAAGTGAACGG
3	RM 411	ACACCAACTCTTGCCTGCAT	TGAAGCAAAAACATGGCTAGG
8	RM 433	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC
9	RM 444	GCTCCACCTGCTTAAGCATC	TGAAGACCATGTTCTGCAGG
10	RM 484	TCTCCCTCCTCACCATTGTC	TGCTGCCCTCTCTCTCTCTC
8	RM 506	CGAGCTAACTTCCGTTCTGG	GCTACTTGGGTAGCTGACCG

3	RM 517	GGCTTACTGGCTTCGATTTG	CGTCTCCTTTGGTTAGTGCC
2	RM 535	ACTACATACACGGCCCTTGC	CTACGTGGACACCGTCACAC

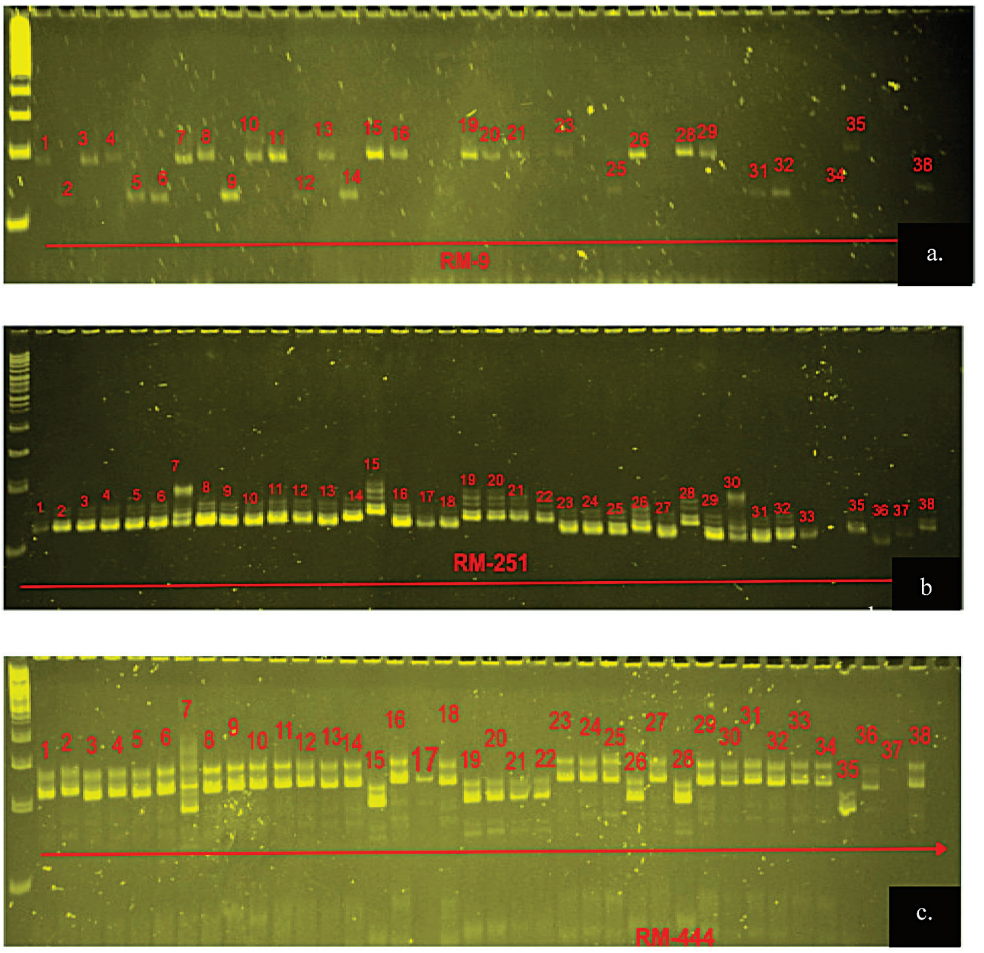

Fig. 1. SSR primers banding pattern of thirty eight aromatic rice landraces using primers a) RM 9 b) RM 251 and c) RM 444

Sequence of 38 rice landraces in gel pictures

1= Tulsimanjari	2= Kapoorsar	3= Kalikamod	4= Atmasheetal	5= Jiradhan	6= Chinnor-1
7= Chinnor-2	8= Shukulaphool	9= Elaychi	10= Gangabaru	11= Jaigundhi	12= Anterved
13= Tulsiprasad	14= Kubrimohar-1	15= ubrimohar-2	16= Keraghul	17= Samudrafan	18= Jouphool
19= Dubraj-1	20= Dubraj-2	21= Dubraj-3	22= Maidubraj	23= Lallu-14	24= Katarnibhog
25= Badshahbhog	26= Karigilas	27= Shrikamal	28= Tilkasturi	29= Vishnubhog-1	30= Vishnubhog-2
31= Shyamjeera	32= Bisni	33= Jeeraphool	34= Londhi	35= Dujai	36= Gopalbhog
37= Jawaphool	38=Kasturi				

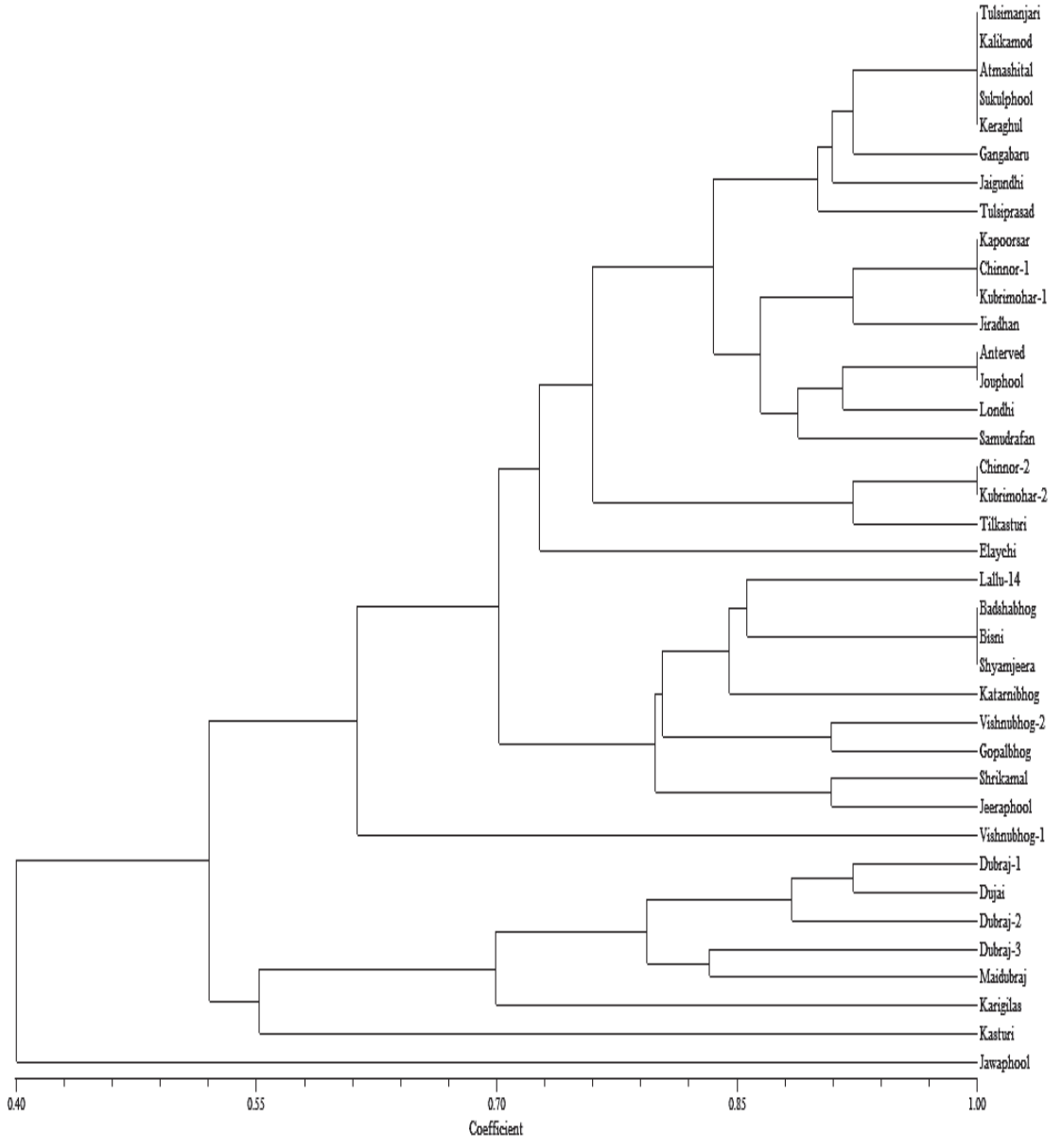

Fig. 2. Dendrogram showing clustering pattern of thirty eight rice cultivars based on 19 SSR primers using UPGMA method