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MOLECULAR CHARACTERIZATION OF AROMATIC RICE, *ORYZA SATIVA* L. USING SIMPLE SEQUENCE REPEATS (SSR) 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. Out of 19, 13 SSR primers revealed polymorphism and 3 primers exhibited monomorphism reaction while the remaining 6 primers showed no reaction. The SSR data was analyzed to determine the genetic similarity coefficients, which 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, SSR, polymorphism.

Introduction

Rice (*Oryza sativa* L.) feeds more than 50% of the tropical populace. Though a high-volume, low-value commodity, 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 the domestic as well as international markets. India has become a leading exporter of aromatic rice steadily exporting 0.5-0.6 million tons of Basmati rice and 1.5-2.5 million tons of non Basmati rice contributing Rs. 3000-4000 crores to the Indian economy. With growing demand for aromatic rice in international market high emphasis was placed till now on improvement of basmati types. The improvement of indigenous small and medium grained aromatic rice, which possess outstanding quality like aroma, kernel elongation after cooking, fluffiness and taste were 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.

Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programmes and 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.

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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. Thirty eight aromatic land races of rice from Chhattisgarh were selected for this study (table 1). Healthy seeds of each variety were sowed in soil pots containing water under appropriate growth conditions for getting fresh leaves. Total genomic DNA was extracted out from six weeks old rice seedlings of each of the landraces of rice

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

S. no.	Variety	Sources	S. no.	Variety	Sources
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

by mini prep method of DNA extraction. Weigh around 0.1g of leaf sample and put it into a 2ml eppendorf. Add 0.4ml of extraction buffer and beats. Grind in a crusher. Add 0.4ml of Chloroform-isoamyl alcohol mixture, mix well by vortexing. Centrifuge at 14000 rpm for 4 min collect the supernatant and transfer to a new eppendorf tube (Repeat). Add 0.8ml of absolute ethanol and mix properly by the tube inversion. Centrifuge at 14000 rpm for 4 min. Discard the supernatant and wash the pellet with 70% Ethanol. Dry the pellet for 15-20 min. Dissolve the pellets in 20-40 µl (based on the size of the pellet) of TE Buffer or Double Distilled Sterile water. Treat with 3 µl RNase for 20 min to remove RNA.

Quantification of DNA

The nano drop quantification was performed for this research work.

Nano drop spectrophotometer based

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 as 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.

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:

$$\text{Dilution} = \frac{\text{Required conc. of DNA (ng/}\mu\text{l)} \times \text{Total volume required (}\mu\text{l)}}{\text{Available conc. of crude DNA (ng/}\mu\text{l)}}$$

SSR analysis

For the SSR analysis of rice landraces 2 µl diluted template DNA of each of the aromatic rice entry was dispensed in PCR plates. Separate cocktail was prepared in an eppendroff tube. The quantity of 18µl cocktail was added to each PCR plates having template DNA.The reaction was carried out in 20 µL reaction volume containing 2 µL 10 nenogram/ µL genomic DNA, 11 µL nanopure water, 2 µL 10 × 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 cycles. Amplification was performed in a thermal cycler with an

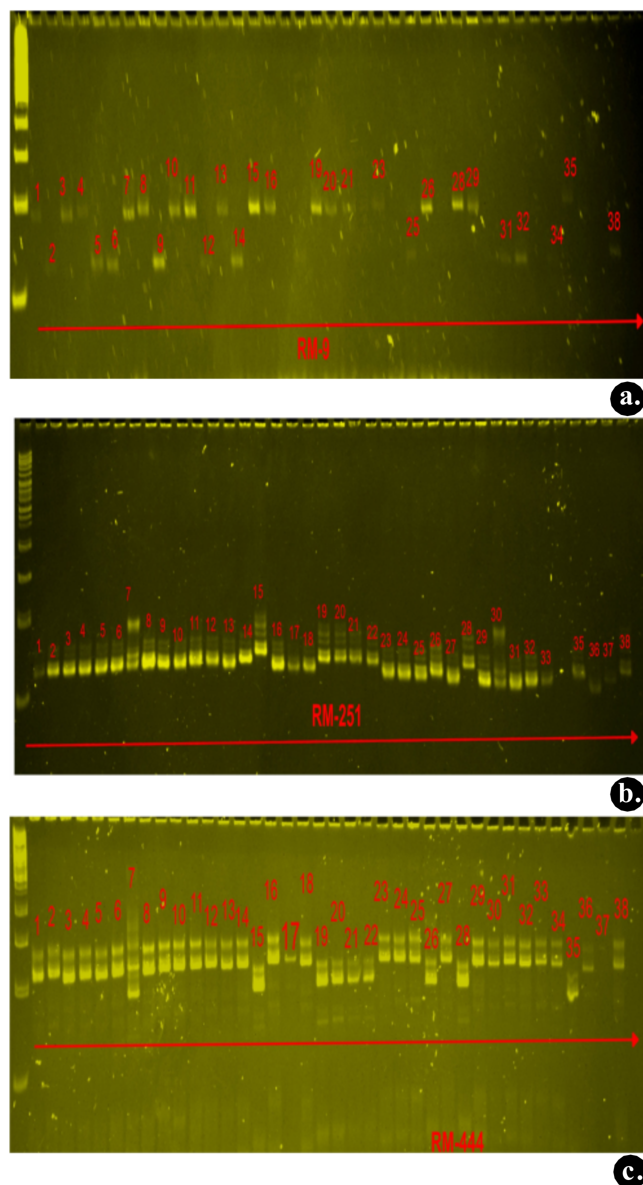

Fig. 1 : SSR primers banding pattern of thirty eight aromatic rice landraces using primers **a)** RM 9 **b)** RM 251 and **c)** RM 444.

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 = Kubrimohar-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.

initial denaturation of 94^{0}C for 4 min followed by 35 cycles which contains denaturation at 94^{0}C 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^{0}C 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 1 x TBE buffer, ladder PBR-322 molecular marker was also loaded along with the DNA samples. Electrophores is done for 1hr at 199 volts. The gel along with the DNA sample then stained with Eithidium bromide (10 µg/10ml) for 40-45 mins. Gel was visualized on UV-transilluminator and image can be seen in computer. The banding pattern of population developed by each set of primer 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) presence of band in a particular base pair position was scored as "1" and absence of band that particular base pair position was scored as "0"(zero). The 19 SSR primers used for this purpose are presented in table 2.

Data analysis

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as "1" and "0" respectively. From the binary data, the similarity coefficient values between the cultivars were derived based on the probability that a particular character of one accession will also be present in another with the Jaccard's correlation analysis using the statistical software "SPSS" version 7.5 for Windows. The statistical analysis is performed using NTSYSpc version 2.1 (Rohlf, 1998). The data matrix was used to construct a phenetic dendrogram using UPGMA (unweighted pair group method of arithmetic averages) (Sneath and Sokal, 1973) in order to cluster the accessions.

Results and Discussion

The results of present study indicated a considerable level of genetic diversity among the cultivars selected. In total 19 SSR markers were used to study the diversity of genotypes, out of which 13 SSR markers produced results whereas the results from 6 markers were not clear. Nineteen SSR markers named 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 situated on different rice chromosomes were studied. Six of which (Rm 228, Rm 245, Rm 302, Rm 307, Rm 323 and Rm 517) do not respond clearly with the landraces under study. 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

Table 2 : Microsatellite markers used for genetic diversification among 38 rice land races.

Chromosome numbers	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	TGGAGAAGATGGAATACTTGC
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

markers Rm 215, Rm 288 and Rm 506 exhibited monomorphic reactions (fig. 1). On the basis of these thirteen SSR markers or more specifically thirteen locus study the genetic diversity or genetic similarity were studied. The dendrogram derived from UPGMA cluster analysis based on of 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 there are two major groups were formed having 40% similarity *i.e.* Jawaphool with rest of the aromatic landraces. All the aromatic landraces except Jawaphool were again grouped into two classes at nearly 50% genetic similarity level. One group contains Dubraj-1, Dubraj-2, Dubraj-3, Maidubraj, Kharigilas, Kasturi and Dujai and other group includes 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.

In group I Kasturi, which is not a aromatic landrace of Chhattisgarh exhibited 55% similarity with Dubraj group, Dujai and Kharigilas. Kharigilas and rest of the races of group I having 70% genetic similarity. Further 5 landraces of group I forming two classes, which indicated that Dubraj-3 and Maidubraj have genetic similarity nearly 82%, whereas Dubraj-2 is nearly 88% similar with Dubraj-1 and Dujai. Dubraj-1 and Dujai having 93% genetic similarity according to 13 locus under study.

In II group Vishnubhog-1 exhibited 61% genetic similarity with rest of the entries of group II. This group is 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. On the basis of 13 loci analysis of SSR markers, Tulsimanjari, Kalikamod, Atmasheetal, Shuklaphool, Kheraghul showed 100% similarity whereas 91% similarity with Gangabaru.

Kapoorsar, Chinnor-1 and Kubrimohar exhibited 100% genetic similarity for loci under study. Whereas, exhibited 91% genetic similarity with Jeeradhan. 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

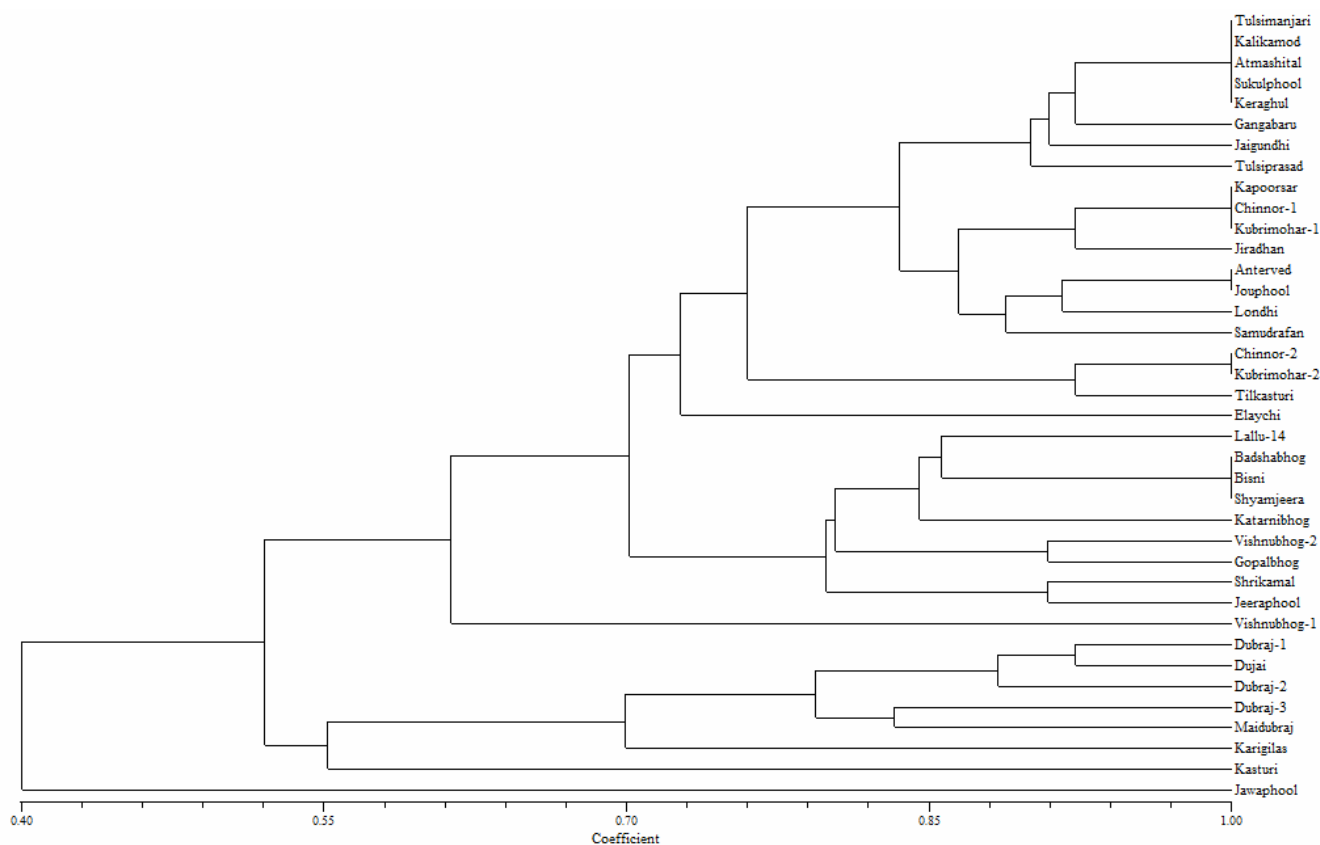

Fig. 2 : Dedogram showing clustering pattern of thirty eight rice cultivars based on 19 SSR primers using UPGMA method.

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*.

Conclusion

The present study revealed that the identification of genotypes and to maintain its genetic purity, knowledge of different morphological markers is very essential. This information will be very useful for the persons involved in seed certification and seed production programme. The polymorphism detected among the landraces will be helpful in selecting genetically diverse cultivars in future breeding programme. However, there were some precincts in the present study that only thirty eight landraces and nineteen primers were used in SSR analysis and hence reduce the chance to obtain a reliable knowledge precisely about the genetic structure of each cultivar of rice. Further studies involving large number of accessions and primers need to be conducted to get more precise information.

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