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Full Length Research Paper

Identification and differentiation of indigenous non-Basmati aromatic rice genotypes of India using microsatellite markers

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Aromatic rice is preferred by consumers all over the world due to its flavor and palatability. Although large number of them is available, little analysis of the genetic diversity has been done at molecular level so far. Twelve microsatellite primer pairs, one from each chromosome of rice were used for evaluating the genetic diversity of 38 traditional indigenous non-Basmati aromatic rice cultivars. A total of 32 different reproducible bands were amplified of which 26 (81.25%) were polymorphic. The number of bands per primer ranged from one to six with an average of 2.6 bands per primer. Ten primers (83.3%) revealed polymorphism between cultivars. Polymorphism information content ranged between 0.00 to 0.83. A dendrogram based on cluster analysis by microsatellite polymorphism grouped all the 38 aromatic rice genotypes into three major groups effectively differentiating the slender aromatic rice cultivars from the short bold and long bold aromatic cultivars. Interestingly, Katrani, medium slender aromatic rice from Bihar had to be grouped separately being genotypically different from other cultivars. It could be concluded that microsatellite markers could efficiently identify indigenous non-Basmati aromatic rice genotypes which can help in genetic conservation management and support intellectual property protection.

Key words: *Oryza sativa*, microsatellite, genetic diversity.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crop in the world, growing in over 1.5 billion hectares of land having overall worldwide production of 596 million tons per annum (FAO, 1999). Now, rice is being grown in 117 countries and is a staple food for more than one half of the global population. India alone produces nearly one fourth (22%) of the rice in the world, next only to china (Herdt, 1991). It is one of the very few crop species endowed with rich genetic diversity which account over 100,000 landraces and improved cultivars and makes it one of the most researched crop with wealth of scientific literature on all its aspects. Most of the information we have so far is about common varieties, and our knowledge on aromatic rices is still incomplete. An ever increasing global demand for aromatic rice has been noted in the recent times. Chaudhary et al. (2003) have discussed the economic aspects of aromatic rice detailing current trends, consumption pattern and global market demands.

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 tonnes of Basmati rice and 1.5 - 2.5 million tonnes 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 possesses 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. Aroma, elongation and taste of Bindli, short grained aromatic rice is known to be superior to Basmati

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types. Domestic market exists for the indigenous aromatic rice which are popular in their native areas of cultivation. Little attention has been paid to their improvement except for sporadic reports on germplasm evaluation and genetics of some quality trait. As such there is very little information available on genetic diversity of traditional non-basmati indigenous aromatic rice. With introduction of high yielding varieties, the land races that include aromatic quality types are moving out of cultivation. Therefore, these varieties have to be collected and evaluated for their exploitable genetic variability and conserved. Further, management of the indigenous aromatic rice genetic resources by way of characterisation and documentation helps in protection of these unique bioresources in accordance with the provision laid out in the (1992) meet on Conservation of Biological Diversity (CVT). A large collection of aromatic rice is available at several national and international Institutes including Central Rice Research Institute, Cuttack, India.

Traditionally used morphological and biochemical markers have not been found to be discriminative enough, warranting more precise techniques. Further, these markers are not reliable because many characters of interest have low heritability and are genetically complex. Several molecular marker techniques are now available. They are more reliable, and remain unaffected across different growth stages, seasons, locations and agronomic practices. In fact, the restriction fragment length polymorphism (RFLP) approach has been used successfully to identify genetic markers in plants, including rice (Tanksley et al., 1989). However, the RFLP technique needs specific probes for the target DNA sequences, and use of radioactive elements makes it more costly and tedious. The development of Polymerase Chain Reaction (PCR) based techniques has offered a good alternative to the RFLP analysis. The PCR based randomly amplified polymorphic DNA (RAPD), microsatellite and amplified fragment length polymorphism (AFLP) approach requires less DNA, and is technically simple, quicker and cheaper compared to the RFLP (Welsh et al., 1990; Williams et al., 1990). These markers have been recently utilised for many purposes including genome mapping, gene tagging, estimation of genetic diversity, varietals differenttiation, resolution of uncertain parentage and purity testing (Mc Couch et al., 1997; Olufowote et al., 1997; Bligh et al., 1999; Coburn et al., 2002; Ni et al., 2002). Among PCR based markers, microsatellite markers are highly polymorphic, more reproducible, co-dominant and distributed throughout the genome. More than 2200 microsatellite markers have been mapped to specific locations in rice genome (Mc Couch et al., 2002). A random set of these mapped markers providing genome-wide coverage should facilitate an unbiased assay of genetic diversity and thus giving a robust, unambiguous molecular description of rice cultivars (Nagaraju et al., 2002). The working group on Biochemical and Molecular Techniques (BMT) of the International Union for the Protection of New Varieties of Plants (UPOV) has in fact identified microsatellite as the most widely used marker system for plant variety characterisation (UPOV, 2002). In the present study, a set of 12 microsatellite markers distributed on 12 different chromosomes of rice genome were used for DNA profiling of 35 non-Basmati indigenous aromatic rice genotypes along with 3 basmati rice varieties and to detect genetic diversity within these cultivars at molecular level.

MATERIALS AND METHODS

Plant material

Thirty-five cultivars of indigenous non-Basmati aromatic rice (*Oryza sativa* L.) from different provinces of India were evaluated in this study. Nine varieties were from Orissa, six from West Bengal, two from Bihar, seven from Uttar Pradesh, five from North East, two from Andhra Pradesh, two from Madhya Pradesh and one from Gujarat (collected from Rice gene bank of Central Rice Research Institute, India). Three aromatic varieties, Pusa Basmati 1, Basmati 370 and Taraori Basmati also served as control for determining allelic molecular weight in this study. Twenty seeds per cultivar were germinated in Petri dishes and seedlings were transplanted into individual pots in green house. Four week after transplanting, about two centimeters of leaf from each cultivar was harvested. Total genomic DNA was extracted from the leaf samples using the modified CTAB method of Murray and Thompson (1980).

Microsatellite marker analysis

A set of 12 mapped microsatellite markers distributed on all the 12 chromosomes (McCouch et al., 1997; Temnykh et al., 2000; McCouch et al., 2002) were used. The number of markers per chromosome was one. These microsatellite primers were from commercially available microsa-tellite primer kits (Qiagen Operon Technologies, Almeda, California, USA). Individual PCR amplifications for each microsatellite were performed using the PTC 100 56 V programmable thermal controller (MJ Research, Watertown, Mass.).The PCR protocol involved a total volume of 20 µl reaction mixture (Miniatis, 1989) containing 35 ng of genomic DNA, 1X PCR buffer (pH 8.3), 200 µM dNTP mix, 10 pmol of each of the forward and reverse primers, 2 mM of $MgCl_2$ and 1 U of *Taq (Thermophilus aquaticus)* DNA polymerase (MBI Fermentas). The basic PCR program to amplify DNA was as follows: an initial hot start and denaturing step at 93°C for 3 min followed by 35 cycles of a 1 min denaturation at 93°C, a 1 min annealing at appropriate temperature (55°C or 67°C depending on the primer), and a 1 min primer elongation at 72°C. A final extension step at 72°C for 5 min was performed.

Gel electrophoresis

A 10 µl aliquot of the amplified microsatellite samples was combined with 2 µl of a loading buffer (0.4% bromo-phenol blue, 0.4% xylene cyanole and 5 ml of glycerol) and was analyzed directly on 2.5% agarose gels in 0.5X TBE buffer (Miniatis, 1989). Electrophoresis was done for about 3 h at 60 volts. 50 bp ladder (MBI Fermentas) was used to compare the molecular weights of amplified products. Visualization of the amplified bands was done by staining with ethidium bromide for 20 min and de-staining with double distilled water for 20 min followed by transillumination under short wave UV light (Alpha Innotech).

Data analysis

Polymorphic products from microsatellite analyses were scored qualitatively for presence (1) and absence (0) for each marker allele-genotype combination. The data entry was done into a binary data matrix as discrete variables.Most informative primers were selected based on the extent of polymorphism. The polymorphic information content (PIC) was calculated by applying the formula given by Powell et al. (1996) and Smith et al. (1997):

$$PIC = 1 - \sum_{i=1}^{n} f^2_{ij}$$

Where f_{ij} is the frequency of j^{th} allele for marker i and the summation extends over n alleles. PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles each in equal and low frequency) A DNA fingerprinting graph was constructed based on the fragments obtained with 12 microsatellite primers. The dendrogram was constructed from the genetic distance matrix obtained by the Euclidean square distance complete linkage using the NTSYS-PC program (Rohlf, 1989).

RESULTS

Band (allele) analysis

Twelve microsatellite primer pairs, one from each chromosome were used for molecular marker analysis of 38 traditional indigenous aromatic rice cultivars (Table 1). A total of 32 different reproducible bands (alleles) were amplified. The number of bands per primer ranged from one (RM259) to six (RM154) with an average of 2.6 bands per primer. The size of the amplified product varied from 125 to 1050 bp. Ten primers (83.3%) revealed polymorphism between cultivars. DNA polymorphism as revealed by one of the highly polymorphic primer is depicted in Figure 1. Of the 32 bands scored, 26 (81.25%) were found to be polymorphic. Maximum number of polymorphic bands i.e. 6 was obtained with the primer RM154 while the primers RM259 and RM230 failed to reveal any polymorphism. The average number of polymorphic bands per primer was 2.10. The polymorphism information content (PIC) ranged from 0.00 (RM259 and RM230) to 0.830 (RM426) with an average of 0.540 (Table 2).

Based on the level of polymorphism detected by individual primers, four most informative primers (RM154, 241, 426 and 225) were identified. These primers amplified a total of 17 bands of which 14 (82.3%) were polymorphic. Two primers, RM259 and RM230 amplified all the bands (alleles) in all the 38 cultivars. Three primers, RM154, RM426 and RM478 failed to amplify all the bands (alleles) in the cultivars Dhusara and Kalanamak 3320; Jaiphool, Kalodhan and Koibra; Laxmibhog, Randhunipagal and Jeeringa Samba, respectively. The cultivars Kalajeera and Chini Kapoor gave the highest number of bands (alleles) (i.e. 23) while the cultivar Kalanamak 3320 gave the least number of bands (alleles) (i.e. 14). RM154 amplified 6 alleles, all being polymorphic (Figure 1). The number of bands varies in between 1 to 4, but the most of cultivars had 3 to 4 bands. Three bands (190, 250 and 305 bp) were found in all varieties except Dhusara and Kalanamak 3320. The band (350 bp) was found in 7 varieties (Haldigundi, Lakhanbhog, Randhunipagal, Gopalbhog, Chakho Uperion, Koibra and Chakhi Chameli). Band (650 bp) was found in 29 varieties. RM241 generated 5 bands of which 2 bands (650 and 700 bp) while three bands (150, 300 and 1050 bp) were monomorphic. Twenty five cultivars amplified only these three monomerphic bands. In each cultivar, the numbers of bands vary from 3 to 5. The band (650 bp) was found in only 6 varieties (Basna-dhan, Thurunbhog, Pimpudibasa, Lakhanbhog, Kalanamak 3315, Koibra and Pusa Basmati 1) whereas band (700 bp) gave amplification in 7 cultivars (Basnadhan, Dhusara, Kalajeera, Pimpudibasa, Katrani, Chini Kapoor and Tulsi Amrit). The primer RM259 and RM230 amplified 1 and 2 alleles, respectively, all being monomorphic. RM426 amplified 3 polymorphic alleles. However, 4 varieties (Jaiphool, Chini Kapoor, Chakho Uperion and Basmati 370) did not show any of the alleles. RM478 ampli-fied 2 alleles, both being polymerphic. Allele (200 bp) was found in 35 varieties while allele (300 bp) was found in only 3 cultivars (Kalajeera, Chini Kapoor and Taraori Basmati).

Cluster analysis

Cluster analysis based on Euclidean^2 distance compete linkage dendrogram classified aromatic rice genotypes into three major groups; I, II and III (Figure 2). First group consisted of 21 long slender and two short slender aromatic rice cultivars and second group consisted of 9 short bold, 3 medium bold and 2 long bold aromatic cultivars. However, the III-group consisted of only one medium slender rice genotype, Katrani from Bihar. The first two major groups were further sub-grouped. In I-group, two major clusters were evident (IA and IB), of which first one was further sub-clustered into three (IA-a, IA-b and IA-c) while second one was sub-clustered into five (IB-a, IB-b, IB-c, IB-d and IB-e). IA cluster consisted of 5 genotypes (Basnadhan, Jala, Chini Kamini, Kalajeera and Pimpudibasa) from Orissa, 4 genotypes (Sitabhog, Randhunipagal, Gopalbhog and Sugandha) from West Bengal and one genotype i.e. Govindabhog from Uttar Pradesh. IB group consisted of 13 genotypes. Infact, genotypes under IB-b cluster (Kalanamak 3315, Adamchini, Kali Komad, Taraori Basmati, Kariga Javelli and Basmati 370) showed a higher degree of similarity. II-group consisted of three major clusters (IIA, IIB and IIC). IIC grouped 2 genotypes (Kalodhan and Improved Raskadam) and IIB grouped 3 genotypes (Lakhanbhog, Chakho Uperion and Koibra). IIA was further sub-clustered into two (IIA-a and IIA-b) with a total of 9 aromatic genotypes. The III-group consists of only one medium slender rice genotype, Katrani from Bihar, which showed significant difference from other two groups. It might be due to very specific characteristics present in Katrani but absent in remaining 37 aromatic cultivars.

All the rice genotypes used in the present study could

Table 1. List of aromatic rice cultivars used for microsatellite analysis.

Sample No.	Name	GrainCharacter[A]	State of India	Source[B]
1	Basnadhan	LS	Orissa	CRRI, Cuttack
2.	Jala	LS	Orissa	CRRI, Cuttack
3	Chini Kamini	LS	Orissa	CRRI, Cuttack
4	Haldi Gundi	LS	Orissa	CRRI, Cuttack
5	Dhusara	LS	Orissa	CRRI, Cuttack
6	Thurunbhog	SB	Orissa	CRRI, Cuttack
7	Jaiphool	SB	Orissa	CRRI, Cuttack
8	Kalajeera	LS	Orissa	CRRI, Cuttack
9	Pimpudibasa	LS	Orissa	CRRI, Cuttack
10	Lakhanbhog	SB	West Bengal	CRRI, Cuttack
11	Laxmibhog	MB	West Bengal	CRRI, Cuttack
12	Sitabhog	LS	West Bengal	CRRI, Cuttack
13	Randhunipagal	LS	West Bengal	CRRI, Cuttack
14	Gopalbhog	LS	West Bengal	CRRI, Cuttack
15	Krishnabhog	SB	West Bengal	CRRI, Cuttack
16	Katrani	MS	Bihar	CRRI, Cuttack
17	Sugandha	LS	Bihar	CRRI, Cuttack
18	Tilak Chandan	SB	Uttar Pradesh	CRRI, Cuttack
19	Bindli	SB	Uttar Pradesh	CRRI, Cuttack
20	Kala Namaka 3315	LS	Uttar Pradesh	CRRI, Cuttack
21	Kala Namaka 3320	LS	Uttar Pradesh	CRRI, Cuttack
22	Adam Chini	LS	Uttar Pradesh	CRRI, Cuttack
23	Govindabhog	SS	Uttar Pradesh	CRRI, Cuttack
24	Chini Kapoor	LS	Uttar Pradesh	CRRI, Cuttack
25	Kalodhan	LB	North East	CRRI, Cuttack
26	Chakho Uperion	MB	North East	CRRI, Cuttack
27	Koibra	MB	North East	CRRI, Cuttack
28	Chakho	SB	North East	CRRI, Cuttack
29	Chakhi Chameli	LS	North East	CRRI, Cuttack
30	Kariga Javelli	SS	Andhra Pradesh	CRRI, Cuttack
31	Sapri 17	LS	Madhya Pradesh	CRRI, Cuttack
32	Jeeringa Samba	SB	Andhra Pradesh	CRRI, Cuttack
33	Pusa Basmati 1	LS	Premium Basmati	CRRI, Cuttack
34	Kali Komad	LS	Gujarat	CRRI, Cuttack
35	Improved Raskadam	LB	West Bengal	CRRI, Cuttack
36	Tulsi Amrit	SB	Madhya Pradesh	IARI, New Delhi
37	Basmati 370	LS	Premium Basmati	IARI, New Delhi
38	Taraori Basmati	LS	Premium Basmati	IARI, New Delhi

[A]Grain character abbreviations: SS = Short slender, LS = long slender, MS = medium slender, SB = short bold, LB = long bold, and MB = medium bold.
[B]Source abbreviation: CRRI, Central Rice Research Institute, Cuttack-753006, India; IARI, Indian Agricultural Research Institute, Pusa, New Delhi-1100001, India.

primers individually was so informative as to differentiate all the genotypes, highly polymorphic profiles were obtained with four of the primers, RM154, RM519, RM13 and RM222. The frequency of the polymorphic fragments detected with these primers ranged from 7/38 to 36/38. In particular, the combination of the polymorphic fragments and monomorphic alleles obtained with all the 12 primers enabled the development of DNA fingerprints of the rice genotypes (Figure 3).

DISCUSSION

In the present study, microsatellite profiling was found efficient enough to reveal usable level of DNA polymerphism among indigenous non-Basmati rice genotypes. This

Table 2. Details of SSR markers used, indicating their location of rice chromosomes, number of alleles and polymorphism information content (PIC) detected.

Chromosome	Position (cM)	Primer name	No. of alleles	No. polymorphic alleles	PIC[A]
1	54.2	RM259	1	0	0.00
2	4.8	RM154	6	6	0.44
3	157.3	RM426	3	3	0.83
4	106.2	RM241	5	2	0.33
5	28.6	RM13	2	2	0.73
6	26.2	RM225	3	3	0.73
7	93.8	RM478	2	2	0.57
8	112.2	RM230	2	0	0.00
9	3.3	RM444	2	2	0.73
10	11.3	RM222	2	2	0.72
11	120.1	RM224	2	2	0.65
12	62.6	RM519	2	2	0.75
		Total	**32**	**26**	**0.54 (Av.)**

[A]PIC, Polymorphism information content

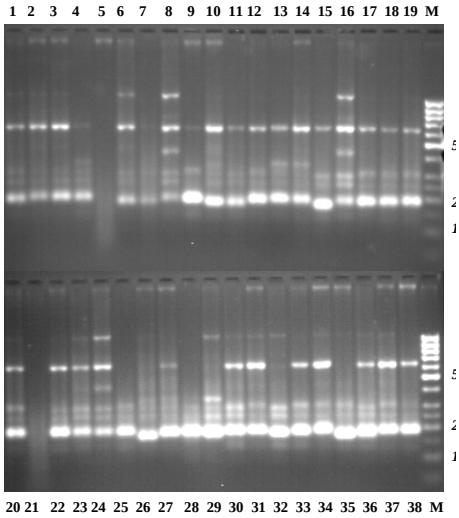

Figure 1. Microsatellite profiles of aromatic rices obtained with primer RM 154. M = Molecular weight marker (50 bp DNA ladder). Numbers on the top and bottom of the lanes correspond to genotypes given in Table 1. Numbers on the right margin represent molecular weight markers in bp.

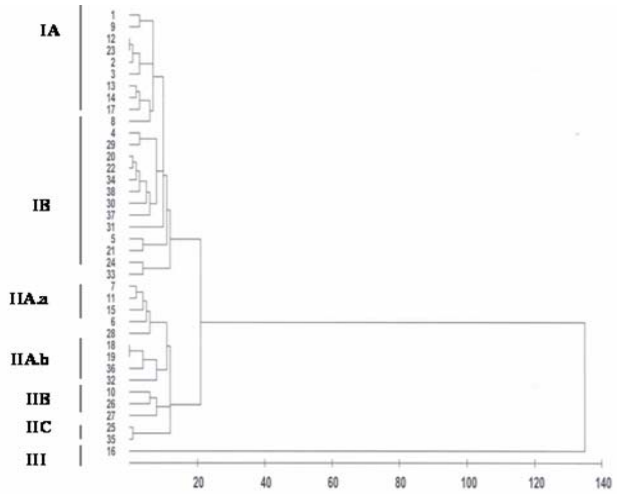

Figure 2. Euclidean^2 distance complete linkage dendrogram. Numbers in the left hand margin represent aromatic cultivars. Scale in the bottom represents the genetic distance. Major cluster and sub-cluster are indicated on left margin. Numbers in the left correspond to genotypes, given in Table 1.

RAPD profiling (Fukaoka et al., 1992; Virk et al., 1995a; Choudhury et al., 2001) and microsatellite profiling (Singh et al., 2004) in rice. The average polymorphism information content (PIC), 0.54 obtained is higher than the earlier observations (Sebastian et al., 1998; Singh et al., 2004). However, it correspond well when less number of microsatellite loci are considered (Garland et al., 1999). The number of alleles detected by microsatellite primers varied from 1 to 6 with an average of 2.6 alleles per primer. The number of alleles detected in the present study showed higher level of allelic diversity with the earlier report (Sebastian et al., 1998; Singh et al, 2004).

This may be due to inclusion of more number of non-Basmati aromatic rice cultivars. Three primers, RM154, RM426 and RM478 failed to amplify all the bands (alleles) in the cultivars, Dhusara and Kalanamak 3320; Jaiphool, Kalodhan and Koibra; Laxmibhog, Randhunipagal and Jeeringasamba, respectively. This is because all these genotypes might have similar sequence at the specific locus of the chromosome.

Cluster analysis based on Euclidean^2 distance complete linkage classified 38 aromatic rice genotypes into 3 major groups. Distinct distribution of short bold from long slender aromatic rice corresponded well with their geographic distribution as well as grain characteristics. The

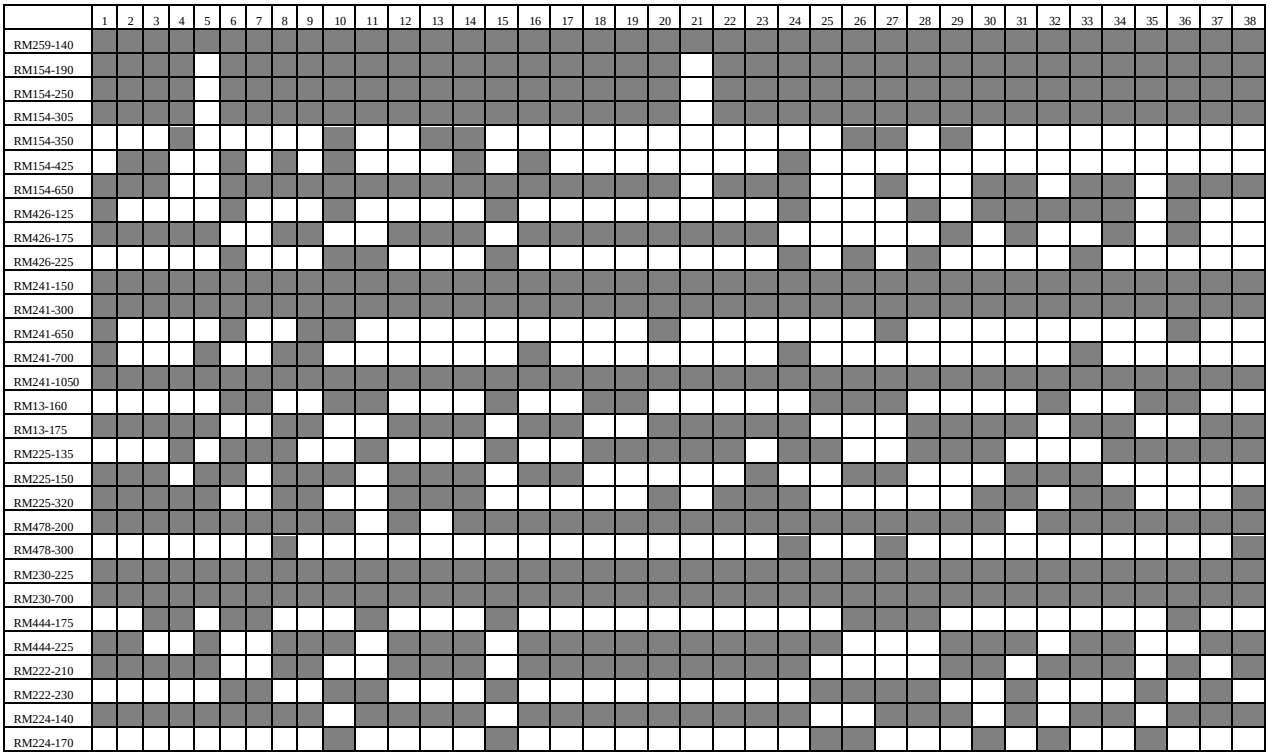

Figure 3. Diagrammatic presentation of DNA fingerprints. Each column corresponds to a cultivar (Table 1) and each row represents the fragment pattern in respect of a marker across the cultivars. For instance the first row (RM259 -140) represents the pattern of a 140 bp fragment, which was obtained with primer RM259. The shaded region corresponds to the presence of bands.

long slender and medium slender aromatic rice are largely grown in North and North- Western parts of India like Uttar Pradesh and Gujarat and possess most desirable grain characteristics and cooking qualities which depend upon the combined effect of several physico-chemical properties. However, the short bold rice cultivars included in the present study are grown mostly in Eastern India (Orissa, Bihar and West Bengal) and North-Eastern parts of India. These cultivars, although possess different kinds of aroma with varying strength, lack the more desirable Basmati traits. However, Katrani, the medium slender rice from Bihar acted differently from other slender genotypes. This suggests a probability that the widely accepted long slender basmati types may have developed by natural mutation of some indigenous non-Basmati types.Genetic diversity analysis of a large number of aromatic rice collections employing DNA profiling would involve a great deal of effort, time and cost. On the basis of the observations in the present study, it is suggested that microsatellite analysis can be efficiently utilized for this purpose. Moreover, since the markers were chosen from all the chromosomes of rice, the levels of diversity exhibited by them are likely to be unbiased and not due to chance. So in combination with multiplexing in PCR, these markers can be efficiently utilized for testing purity of the commercial seed lots and for maintenance of seed purity at different levels of seed production of aromatic rice. However, the present study is still at its infancy. The diversity obtained among the rice cultivars accounts for even less than 1% of rice genome. A lot of primers from different chromosomes are required to analyze even much larger germplasm to get a reproducible data that can be used as ready references by concerned breeders, variety registration authority and seed production agencies.

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