



## Identification and classification of aromatic rices based on DNA fingerprinting

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Received 17 August 1999; accepted 7 June 2000

**Key words:** aromatic rice, DNA fingerprinting, genetic relationship, *Oryza sativa*, RAPD

### Summary

Aromatic rices are preferred by the consumers all over the world due to its flavour and palatability. Although a large number of these collections are available, little systematic analysis of genetic diversity has been carried out. With the objective of identification and classification of aromatic rice genotypes, RAPD profiling was employed using 58 random decamer primers. Most of these primers (96.5%) detected polymorphism among the genotypes. Of the 465 amplified bands, 314 were polymorphic. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the traditional tall, photosensitive, low yielding, long grained 'basmati' aromatics together. The short grained aromatic cultivars, formed a different cluster with high level of average similarity among themselves. The dendrogram based on 58 primers was highly similar to that based on 10 and 15 primers with matrix correlation ( $r$ ) of 0.88 and 0.91, respectively. This suggested that a set of 10 primers can be employed for an initial assessment of genetic diversity in a large number of collections. All the rice genotypes included in the study could be distinguished from each other at the level of 19 to 186 polymorphic bands between individuals in pair wise comparison over all the 58 primers. Probability of identical profiles by chance suggested that about  $10^{41}$  genotypes can be unambiguously differentiated by RAPD fingerprints obtained by 58 primers. A diagrammatic mode of presentation of DNA fingerprints of the aromatic rices based on 10 of the informative primers was developed.

### Introduction

Unambiguous, reliable, fast and cost-effective determination of genetic diversity in plant varieties, breeding lines and accessions is essential for effective utilization of plant genetic resources in crop improvement. Morphological characters, both qualitative and quantitative have been employed for estimating diversity. Due to stage specific expression of characters and influence of environment, morphological diversity estimates are less reliable. Moreover, at times there may be little morphological diversity among cultivars with related pedigrees. Use of molecular markers are considered best for analysis of genetic diversity and varietal identification since there is no effect of stage of development, environment or management practices. Availability of a large number of polymorphic markers enables precise classification of the cultivars and ger-

mplasm collections. Of the available molecular marker systems, RAPD technique (Williams et al., 1990) is the fastest and simplest. In rice, RAPD analysis has been used extensively for a variety of purposes which include identification and classification of accessions (Fukuoka et al., 1992; Virk et al., 1995b), identification of hybrids (Qian-Qian et al., 1996), estimation of Sland race. genetic diversity (Yu & Nguyen, 1994; Mackill, 1995; Cao & Oard, 1997) and predicting quantitative variation within germplasm (Virk et al., 1996).

Aromatic rice is an important commercial commodity. It is more preferred by the consumers all over the world because of its scent and palatability. A large collection of aromatic rices is available at several national and international institutes including International Rice Research Institute, Manila, Philippines, and Central Rice Research Institute, Cuttack,

India. This consists of both long grain and short grain cultivars, breeding lines and land races. The Indian aromatic rices greatly vary in grain quality including strength of aroma, length/breadth ratio and cooked kernel elongation ratio. These constitute an important source of genetic variation for utilization in breeding of high yielding aromatic rice varieties and hybrids. A systematic analysis of these collections based on DNA profiling is essential for accurately estimating genetic diversity and identifying the duplicates included as different accessions. There is very little information available on genetic diversity of aromatic rices. Hence, in the present study, the extent of genetic diversity among the long grained elite aromatic rices was determined and their genetic relationship with the short grained aromatic rice cultivars was established. Besides, the possibility of identifying the genotypes based on RAPD fingerprints was demonstrated.

## Materials and methods

### *Plant materials*

Seeds of 48 elite cultivars/breeding lines of aromatic rice (*Oryza sativa* L.) were obtained from rice collections maintained at different research institutes in India like Central Rice Research Institute, Cuttack, Orissa; Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttar Pradesh; Indian Agricultural Research Institute, New Delhi; Regional Agricultural Research Station, Titabar, Assam and Regional Research Station, Majian, West Bengal (Table 1). Seeds were germinated and grown under aseptic condition at about 30 °C.

### *Plant DNA extraction*

DNA was isolated following the protocol of Doyle & Doyle (1990). Freshly germinated young seedlings were bulked and five grams of tissue was ground to a very fine powder in liquid nitrogen and dispersed in 20 ml of pre-warmed (60 °C) CTAB DNA extraction buffer (2% CTAB; 1.4 M NaCl; 20 mM EDTA, p.H. 8.0; 100 mM Tris-HCl, p.H. 8.0; 0.2%  $\beta$ -mercaptoethanol). After incubation for 1 hr at 60 °C with intermittent swirling, the mixture was emulsified with an equal volume of chloroform: isoamyl alcohol (24: 1). Following centrifugation, the aqueous phase was collected and mixed with 0.6 volume of chilled isopropanol by quick inversion. The precipitated nucleic acid was spooled out, washed twice

in 70% ethanol, dried under vacuum and dissolved in 1 ml T<sub>10</sub>E<sub>1</sub> (Tris 10 mM, EDTA 1 mM) buffer. The RNA was removed by RNase treatment at 37 °C for 1 hr. For further purification, DNA solution was extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1; p. H. 8.0) followed by two extractions with chloroform: isoamyl alcohol (24: 1). The upper aqueous phase was separated after centrifugation and mixed with 1/10<sup>th</sup> volume of 3M sodium acetate. DNA was precipitated by adding two volumes of chilled absolute alcohol, pelleted, dried in vacuum and dissolved in T<sub>10</sub>E<sub>1</sub> buffer. Quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gels alongside diluted uncut lambda DNA as standard. DNA was diluted in T<sub>10</sub>E<sub>1</sub> to a concentration of approximately 12.5 ng/ $\mu$ l for use in PCR analysis.

### *DNA amplification*

Arbitrary decamer primers from Operon Technologies Inc., Alameda, CA, USA were dissolved in sterilized T<sub>10</sub>E<sub>1</sub> to a concentration of 15 ng/ $\mu$ l. Fifty-eight primers from Operon kits viz. 19 primers each from A and B series and 20 primers from N series were used for RAPD amplification as described by Williams et al. (1990). Amplification was carried out in a 25  $\mu$ l reaction volume containing 1X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM MgCl<sub>2</sub>), 200  $\mu$ M each of dNTPs, 20 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 25 ng of DNA template. The amplification reaction was carried out in a thermal cycler (Perkin Elmer, Model 9600). The first cycle consisted of denaturation of template DNA at 92 °C for 4 min, primer annealing (37 °C) for 1 min and primer extension (72 °C) for 2 min. In the next 43 cycles, the period of denaturation was reduced to 1 min while the primer annealing and the primer extension time remained as in the first cycle. The last cycle consisted of only primer extension (72 °C) for 8 min.

PCR products were separated on a 1.5% agarose gel containing ethidium bromide using 1X TAE buffer. The size of the amplified fragments were determined by using size standards (100 bp DNA ladder plus or DNA ladder mix, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light and photographed using polaroid photographic system. To test the reproducibility of the profiles, the reactions were repeated at least twice.

Table 1. List of the rice cultivars / breeding lines used in the genetic analysis

Sl.No	Name	Grain character	Source*
1	Pusa Basmati 1	Slender, Long	CRRI, Cuttack, Orissa
2	Karnal Local	Slender, Long	CRRI, Cuttack, Orissa
3	Basmati 370	Slender, Long	CRRI, Cuttack, Orissa
4	Basmati 385	Slender, Long	CRRI, Cuttack, Orissa
5	Taraori Basmati	Slender, Long	CRRI, Cuttack, Orissa
6	Kasturi	Slender, Long	CRRI, Cuttack, Orissa
7	Dehraduni Basmati	Slender, Long	GBPUA & T, Pant Nagar, Uttar Pradesh
8	Pakistani Basmati	Slender, Long	GBPUA & T, Pant Nagar, Uttar Pradesh
9	Basmati (R)	Slender, Long	CRRI, Cuttack, Orissa
10	Basmati (D)	Slender, Long	CRRI, Cuttack, Orissa
11	Gaurav	Slender, Long	CRRI, Cuttack, Orissa
12	PNR 546	Slender, Long	Genetics Division, IARI, New Delhi
13	Chimblate Basmati	Slender, Long	Genetics Division, IARI, New Delhi
14	Seond Basmati	Slender, Long	Genetics Division, IARI, New Delhi
15	Type 3	Slender, Long	Genetics Division, IARI, New Delhi
16	Achhi	Slender, Long	Genetics Division, IARI, New Delhi
17	Muskan	Slender, Long	Genetics Division, IARI, New Delhi
18	Hansraj	Slender, Long	GBPUA & T, Pant Nagar, Uttar Pradesh
19	Haryana Basmati	Slender, Long	Genetics Division, IARI, New Delhi
20	Ranbir Basmati	Slender, Long	Genetics Division, IARI, New Delhi
21	IET 14132	Slender, Long	Genetics Division, IARI, New Delhi
22	Hasan Sarai	Slender, Long	Genetics Division, IARI, New Delhi
23	Tilak	Bold, Medium	Genetics Division, IARI, New Delhi
24	Bindli	Bold, Medium	Genetics Division, IARI, New Delhi
25	BK 843-7	Slender, Long	Genetics Division, IARI, New Delhi
26	RP 3238-33-15-7-1	Slender, Long	Genetics Division, IARI, New Delhi
27	2504-1-3-1	Slender, Long	Genetics Division, IARI, New Delhi
28	1238-95-73-1	Slender, Long	Genetics Division, IARI, New Delhi
29	2504-5-6	Slender, Long	Genetics Division, IARI, New Delhi
30	2503-693-1	Slender, Long	Genetics Division, IARI, New Delhi
31	Basmati Local	Slender, Long	GBPUA & T, Pant Nagar, Uttar Pradesh
32	Tapovan Basmati	Slender, Long	GBPUA & T, Pant Nagar, Uttar Pradesh
33	TTB-196-B-43-2-4-1	Slender, Long	RARS, Titabar, Assam
34	TTB-196-B-43-2-9-1	Slender, Long	RARS, Titabar, Assam
35	TTB-196-B-29-1-23-1	Slender, Long	RARS, Titabar, Assam
36	TTB-196-B-29-1-22-2	Slender, Long	RARS, Titabar, Assam
37	Sonachur	Slender, Short	CRRI, Cuttack, Orissa
38	Tulsi Manjari	Slender, Short	CRRI, Cuttack, Orissa
39	Tulai Pangi	Slender, Short	RRS, Majjan, West Bengal
40	Birendul	Slender, Short	RRS, Majjan, West Bengal
41	Katari Bhog	Slender, Short	RRS, Majjan, West Bengal
42	Randhni Pagal	Slender, Short	RRS, Majjan, West Bengal
43	Kamini Bhog	Slender, Short	RRS, Majjan, West Bengal
44	Gangajali	Slender, Short	RRS, Majjan, West Bengal
45	Leelavati	Slender, Short	RRS, Majjan, West Bengal
46	Chinisakkar	Slender, Short	RRS, Majjan, West Bengal
47	Kalanunia	Slender, Short	RRS, Majjan, West Bengal
48	Sitabhog	Slender, Short	RRS, Majjan, West Bengal

\* CRRI = Central Rice Research Institute.

GBPUA & T = Govind Ballabh Pant University of Agriculture and Technology.

IARI = Indian Agricultural Research Institute.

RARS = Regional Agricultural Research Station.

RRS = Regional Research Station.

### *Analysis of DNA fingerprint patterns*

The RAPD products were scored as present (1) or absent (0) for each primer – genotype combination. The data entry was done into a binary data matrix as discrete variables. Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficients was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The computer package NTSYS-PC (Rohlf, 1990) was used for cluster analysis. Most informative primers were selected based on the extent of polymorphism and the similarity matrices obtained with these primers were compared with that obtained with all the primers using the MXCOMP subprogramme in NTSYS-PC. The average similarity index for all pairwise comparisons ( $\bar{X}_D$ ) were calculated and used to estimate the probability of DNA fingerprints of two cultivars being identical by chance as described by Ramakishana et al. (1994), employing the formula  $(\bar{X}_D)^n$  where,  $\bar{X}_D$  = average similarity index and n = the average no. of amplified products per cultivar.

### **Results**

Fifty-eight decamer primers used in RAPD analysis of 48 aromatic rice genotypes amplified 465 different reproducible bands. The number of bands per primer ranged from three (OPN 4 and OPN16) to 15 (OPA 16), the average bands per primer being 8. The size of the amplified products varied from 0.2 kb to 5 kb. Most of the primers (96.5%) revealed polymorphism between cultivars. DNA polymorphism as revealed by 4 of the highly polymorphic primers is depicted in Figure 1. Of the 465 bands scored, 314 (67.5%) were found to be polymorphic and the rest monomorphic. Maximum number of polymorphic bands (13) were obtained with the primer OPA 16 and the primers OPN 4 and OPN 15 failed to reveal any polymorphism. The average number of polymorphic bands per primer was 5.4. Based on the level of polymorphism detected by individual primers, 15 most informative primers (OPA 11, 12, 13, 16, 17, 19; OPB 1, 5, 8, 11, 13, 17, 18 and OPN 1, 17) were identified. These primers amplified a total of 142 bands of which 112 (78.9%) were polymorphic. When only 10 of the most informative primers were considered (OPA 13, 16, 17, 19; OPB 8, 11, 13, 18 and OPN 1, 17) the percentage of polymorphic bands increased to 82.8%.

Similarity indices estimated on the basis of all the 58 primers ranged from 0.60 to 0.96. The short

grained aromatic genotypes particularly Leelavati, Chinisakkar and Sitabhog were highly similar (average similarity of around 95%). Similarly, high degree of similarity was evident among the aromatic breeding lines 2504 – 1 – 3 – 1, 1238 – 95 – 73 – 1 and 2504 – 5 – 6 as well as between genotypes BK 843 – 7 and RP 3238 – 33 – 15 – 7 – 1. Maximum RAPD diversity was evident between long grained aromatic rice variety PNR 546 and short grained collections such as Kalanunia, Gangajali, Leelavati and Kaminibhog (average similarity of around 60%).

Cluster analysis based on similarity values classified aromatic genotypes into two major groups (I & II, Figure 2). The first major group consisted of seven long grained aromatic rice genotypes whereas, the rest were included in the second major group. Each of these major groups was further sub-grouped. In group one, four of the long grained collections from the North-Eastern India (IB) clustered separately from the long grained aromatic varieties Pusa Basmati 1, Gaurav and PNR 546 (IA). In the second group, two major clusters were evident (IIA & IIB), each of which were further sub-clustered into two. Interestingly, all the short grained genotypes except Kataribhog sub-clustered with high degree of similarity among themselves (IIA2). The commercially important long grained aromatic rice varieties such as Karnal Local, Taraori Basmati, Dehraduni Basmati, Basmati 385 and Basmati 370 were included in a major sub-cluster (IIA1). The classification of rice genotypes based on 58 primers was highly similar with that based on 15 and 10 of the most informative primers with matrix correlation (r) of (+) 0.91 and (+) 0.88, respectively.

All of the rice genotypes used in the present study could be distinguished from each other. Although none of the primers individually was so informative as to differentiate all the genotypes, highly polymorphic profiles were obtained with 10 of the primers such as OPA 13, OPA 16, OPA 17, OPA 19; OPB 8, OPB 11, OPB 13, OPB 18, OPN 1 and OPN 17. The frequency of the 77 polymorphic fragments detected with these primers ranged from 1/48 to 47/48. In particular, the combination of the polymorphic fragments obtained with primers such as OPA 13 (2900 bp), OPA 16 (3200, 3000, 2300, 1650, 750 and 650 bp), OPA 17 (2600, 2100 and 600 bp), OPA 19 (1900, 1025, 615 and 375 bp), OPB 11 (2500, 2000, 1600 and 800 bp), OPN 1 (2000 bp) and OPN 17 (2100 bp) with a comparatively low frequency of occurrence (< 10/48) enabled development of DNA fingerprints of

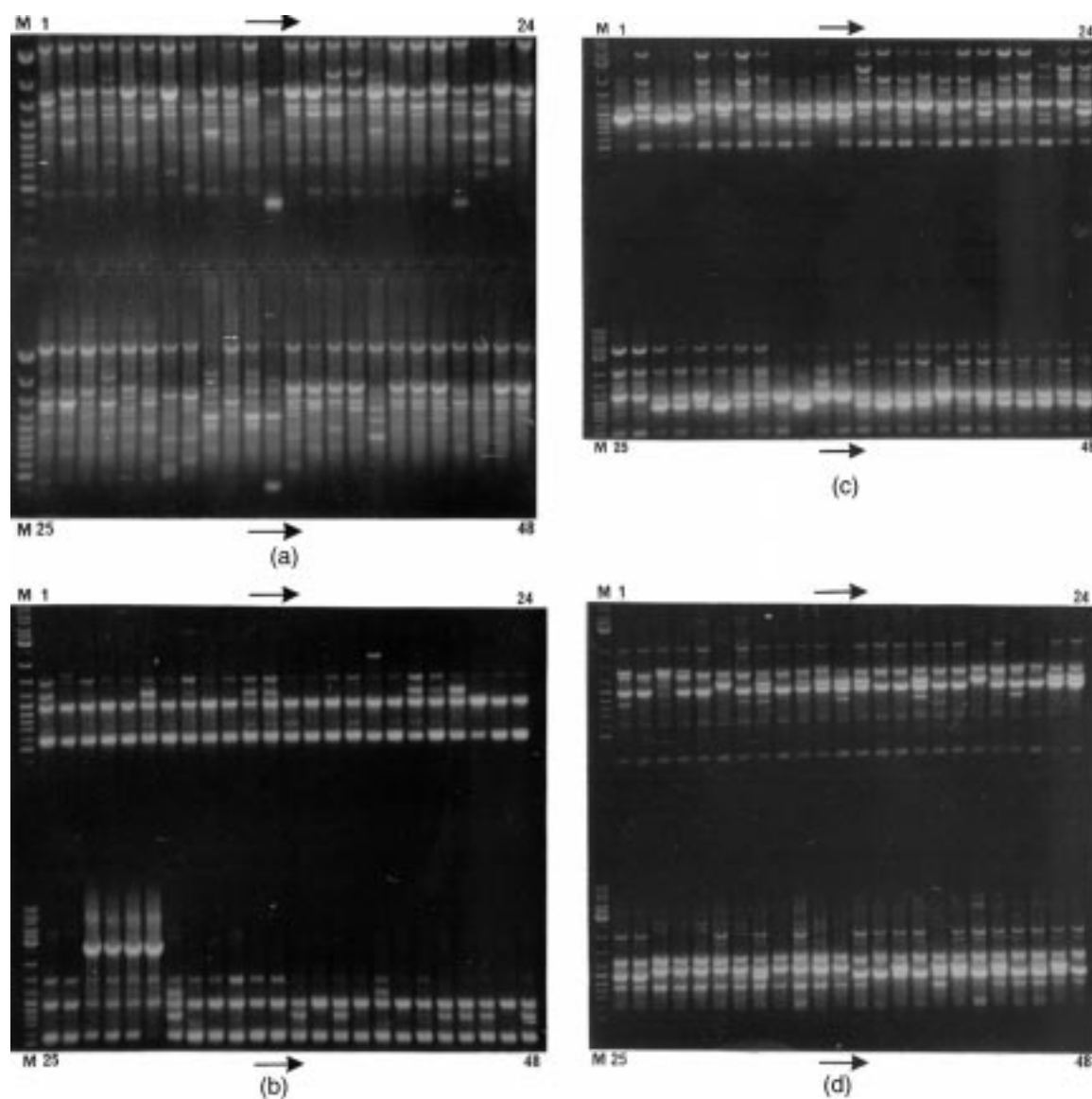


Figure 1. RAPD profiles of aromatic rices obtained with primers OPA 19 (a), OPB 11 (b), OPB 13 (c) and OPB 18 (d). Serial number of the genotypes corresponds to that given in the Table 1. M = molecular weight marker.

Table 2. Analysis of DNA fingerprints using different sets of RAPD primers

	58 Primers	15 Primers	10 Primers
Average bands for each variety $\pm$ SD	$322.3 \pm 12.5$	$83.3 \pm 5.7$	$55.1 \pm 4.0$
Average similarity index ( $\bar{X}_D$ ) $\pm$ SD	$0.747 \pm 0.06$	$0.611 \pm 0.09$	$0.644 \pm 0.10$
Probability of identical match by chance ( $\bar{X}_D$ ) <sup>n</sup>	$1.5 \times 10^{-41}$	$1.1 \times 10^{-15}$	$1.6 \times 10^{-10}$

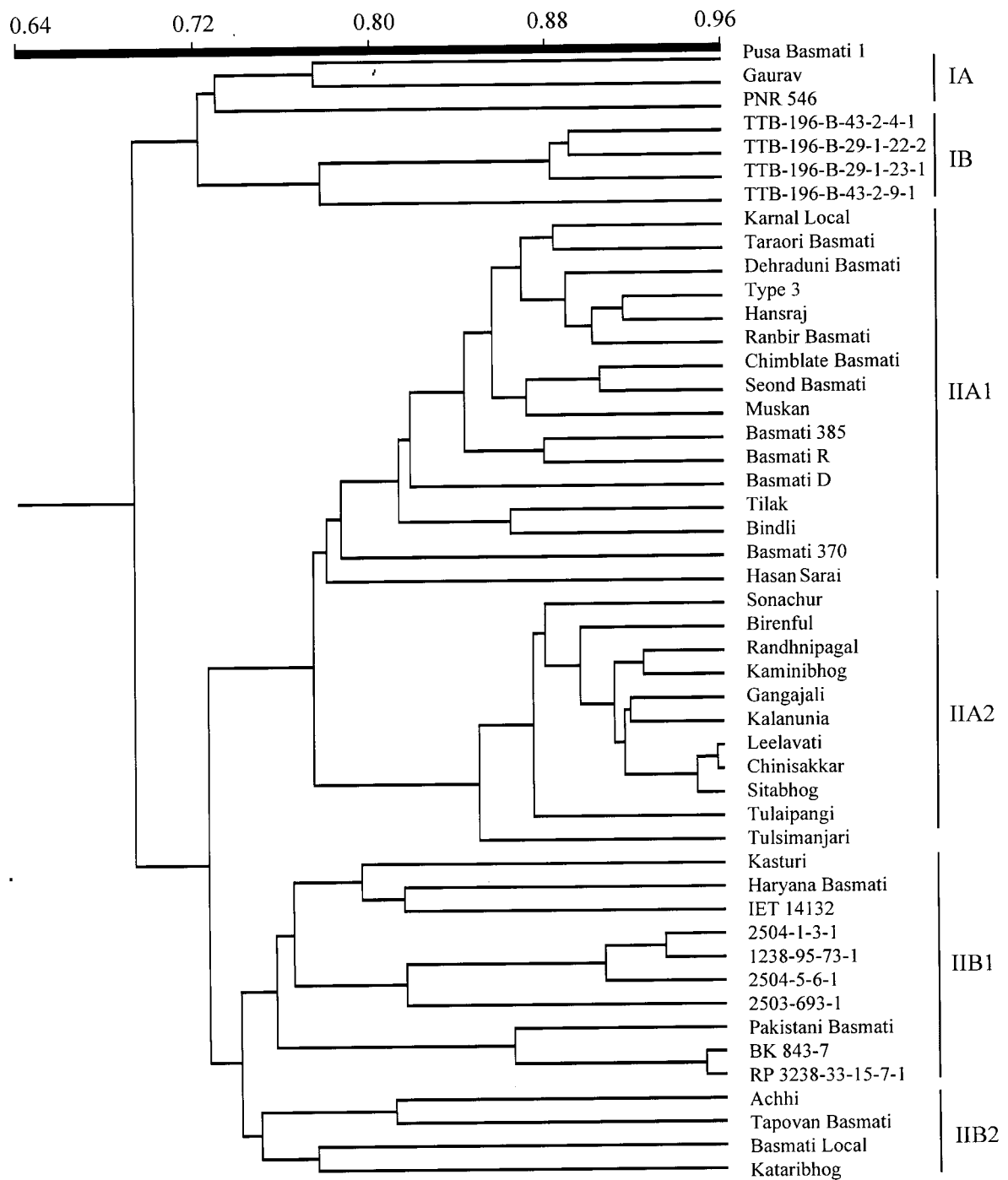


Figure 2. Dendrogram of aromatic rice genotypes, constructed using UPGMA based on 58 RAPD primers. Scale on top is Jaccard's coefficient of similarity. The major clusters and sub-clusters are indicated on right margin.



the rice genotypes which is diagrammatically presented in Figure 3. The probability of identical match by chance was found to be  $1.6 \times 10^{-10}$  based on these 10 most informative primers. This suggests that about  $10^{10}$  aromatic rice genotypes can be distinguished by using these 10 primers. However, by including 5 additional informative primers OPA 11, OPA 12, OPB 1, OPB 5 and OPB 17, the efficiency of the RAPD marker system was increased to the extent that  $10^{15}$  genotypes could be distinguished. Inclusion of all the 58 random primers provided very high resolution power enabling nearly  $10^{41}$  genotypes to be precisely identified (Table 2). Assuming non-overlapping of the amplified products, use of all the 58 primers (with 465 bands, adding up to 666790 bp length) covered approximately 0.16% of the rice genome.

### Discussion

In the present study, RAPD profiling was found efficient enough to reveal usable level of DNA polymorphism among aromatic rice genotypes. This supports the earlier observations with regard to the RAPD profiling in rice (Fukuoka et al., 1992; Virk et al., 1995a), as well as in other crop species (Morell et al., 1995).

The average similarity index of 0.75 between cultivars suggested that the level of genetic diversity among the aromatic rices is low. Minimum similarity was evident between the traditional short grained aromatic cultivars and semi-dwarf high yielding photo insensitive aromatic cultivars such as PNR 546 and Pusa Basmati 1. This can be attributed to the contribution of several genotypes to the development of semi-dwarf aromatic cultivars. Distinct differentiation of tall, photo-sensitive, long grained aromatic rices from the short grained genotypes corresponded well with their geographic distribution as well as grain characteristics. The traditional long grained aromatic rices grown largely in the North-Western part of India possess most desirable grain characteristics and cooking qualities which are determined by a unique and delicately balanced combination of several physico-chemical properties including kernel elongation without significant increase in breadth after cooking, low starch gelatinization temperature, a low to intermediate amylose content and medium gel consistency that are collectively called basmati traits. In contrast, the short grain aromatic rice cultivars included in the present study are grown mostly in Eastern and North-Eastern part

of India. More importantly, these cultivars, although possess different kinds of aroma with varying strength, lack in the more desirable basmati traits. The extent of genetic similarity as determined by the RAPD fingerprinting also corresponded considerably with the known pedigrees. For instance, Taraori Basmati which is a selection from Karnal Local (Ahuja et al., 1995) grouped together with its progenitor parent at similarity index of 0.88. Similarly, Type 3 and Dehraduni Basmati which are considered similar based on morphological features and other traits, clustered together with an similarity index of 0.87. High degree of genetic similarity among the short grain cultivars such as Leelavati, Chinisakkar and Sitabhog (average similarity of >95%) indicated that they are possibly the selections made by the farmers from a single land race.

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 RAPD analysis can be efficiently utilized for this purpose. For an initial survey of diversity, 10 or 15 of the most informative primers presently identified can be used. This seems to be appropriate considering that similarity matrices obtained with these primers highly correlated ( $r = 0.88$  and  $0.91$ , respectively) with that based on, all the 58 primers.

Precise identification of crop cultivars is required for variety registration, preventing misappropriation and for protection of plant breeders' as well as farmers' rights. While the debate is going on with regard to acceptability of DNA data for defining distinctiveness, uniformity and stability (DUS) of crop varieties and deciding on the minimum distance for declaring two varieties as different, creation of data base on the morphological characteristics and DNA fragment patterns is widely accepted by all concerned. In the present study, a basic data set was created for aromatic rices using RAPD fingerprints. The total number of amplified fragments and number of polymorphic fragments contained in this data set enabled identification and differentiation of about  $10^{41}$  aromatic rice genotypes unequivocally. Using a set of polymorphic fragments obtained with 10 of the most informative primers, a diagrammatic mode of presentation of DNA fingerprint has been generated which can serve as a guide for easy visual comparison of any additional genotypes as and when they become available. Besides, this can be obtained for sub-sets of cultivars and distributed to the concerned breeders, variety registration authority, seed production agencies etc. for ready ref-

erence. For declaring two genotypes as different, it is proposed that the percentage of genome covered which can be estimated based on the number and size of the DNA fragments assayed from non-overlapping genomic regions, instead of a single band difference. The aromatic rices included in this study were distinguishable by total genome coverage of approximately 0.16% where, the number of polymorphic fragments between pairs ranged from 19 to 186. This level of distinction is much higher than that proposed by Virk et al. (1995b), who suggested use of 86 to 100 RAPD markers for detecting one or more differences between very similar pair of accessions with 99% confidence. The plant varieties, however, are bred for uniformity in growth and morphological characteristics. It is possible that, different samples of the same variety obtained from different sources might show one or few differences at DNA level, despite their phenotypic uniformity. It is therefore suggested that, instead of setting a limit for the number of markers arbitrarily for variety differentiation at one or few band level, a fixed percentage of genome coverage should be considered.

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