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RAPD profile analysis of betel vine cultivars

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Abstract

RAPD analysis in selected cultivars of Kapoori and Bangla betel vines (*Piper betle* L.) were carried out in order to ascertain the relatedness of the two to each other. On the basis of the data from 10 RAPD primers, it was found that the Kapoori cultivars were more heterogeneous (mean SI = 0.521) while the Bangla cultivars were mostly similar to each other (mean SI = 0.884). Within each type, the overall polymorphism of RAPD bands was more than 70 %. When RAPD band data for both types of cultivars were considered cumulatively, the two were clearly separated from each other. In fact only six bands out of a total of 60 bands were found to be common to cultivars of both types. Bands specific to only one of the two types have potential for developing betel vine cultivar-specific probes and SCAR-markers.

Additional key words: Bangla, heterogeneity, Kapoori, *Piper betle*.

Introduction

Betel vine is an important, traditional and an ancient crop of India. Currently more than 200 cultivars are cultivated in several states of India for its leaves, which are used for chewing purposes. The cultivators and consumers name the cultivars after their localities, villages or towns. Thus the cultivars with prefix Desi in their names invariably refer to the cultivar Bangla in West Bengal, cultivar Kapoori in Maharashtra and cultivar Desavari in Madhya Pradesh (Balasubrahmanyam *et al.* 1995). The traditional nomenclature is thus confusing and attempts to clear the confusion were made. On the basis of phytochemical constituents betel vine cultivars have been divided into five types (Rawat *et al.* 1989). Of these, Bangla and Kapoori are the major types. Morphological and phytochemical characters have been used to distinguish between these two types (Rawat *et al.* 1989). However, these data do not indicate the range and nature of variability in several other characters, amongst the cultivars. Similarly, no molecular work has also been done on characterization of betel vine in India to our knowledge. We have therefore, taken up work on assessing genetic variation amongst the different betel vine cultivars using molecular techniques, in particular, Random Amplified Polymorphic DNA (RAPD; Welsh and McClelland 1990, Williams *et al.* 1990). The present paper describes the results of the study using RAPD technique for showing the distinctness of the Kapoori and Bangla types, and for the determination of extent of diversity within each type.

Materials and methods

Plants: Betel vine (*Piper betle* L., family *Piperaceae*) cultivars were collected from 3 of the centres of the All India Co-ordinated Research Project (AICRP) on Betel vine (Table 1). Young leaf tissue was harvested from the

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Table 1. The betel vine cultivars collected from the different centers for AICRP on betel vine (K - Kapoori type, B - Bangla type, A.P. - Andhra Pradesh, U.P. - Uttar Pradesh, KAR. - Karnataka State in India).

Number	Cultivar	Type	AICRP Center
K 212	Vasani Kapoori	K	Chinthalapudi, A.P.
K 226	Vuyyur Kapoori	K	Chinthalapudi, A.P.
K 228	Sangli Kapoori	K	Chinthalapudi, A.P.
K 214	Pedachapelli Kapoori	K	Chinthalapudi, A.P.
K 218	Dodipatla Kapoori	K	Chinthalapudi, A.P.
K 219	Chinnachapelli Kapoori	K	Chinthalapudi, A.P.
K 238	Bihar Kapoori	K	Chinthalapudi, A.P.
K 208	Cuddappah Kapoori	K	Chinthalapudi, A.P.
K 6	Kapoori	K	Mahoba, U.P.
B 1	Kalkattia Bangla	B	Mahoba, U.P.
B 2	Female Bangla	B	Mahoba, U.P.
B 3	Ayurvedic Bangla	B	Mahoba, U.P.
B 5	Calcutta Bangla	B	Mahoba, U.P.
B 108	Kodwa Bangla	B	Bangalore, KAR.
B 104	Banaras Bangla	B	Bangalore, KAR.
B 7	Desi Bangla	B	Mahoba, U.P.

vines, washed, mopped dry and quickly frozen and powdered in liquid nitrogen. The powders were either used for isolation of DNA immediately, or were stored in a deep freezer (-70 °C) for long-term storage.

Isolation of DNA: Total genomic DNA was isolated from the powdered and frozen young leaf tissue according to the procedure of De Kochko and Hamon (1990) with some modifications (Ranade *et al.* 1997). At least 3 independent DNA preparations were made from leaf tissues collected from each cultivar. The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel.

Polymerase Chain Reaction using RAPD primers: Twenty decamers from kit F and one from kit G (*Operon Technologies*, Alameda, USA) were used as primers. DNA was amplified essentially following Williams *et al.* (1990). Initially a pilot experiment was carried out varying primer, template DNA and Mg^{2+} ion concentrations. The final amplification reactions contained 10 mM N-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS) (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % gelatin, 0.2 mM deoxyribo-nucleotidetriphosphate (dNTP), 10 pmoles primer, 0.5 U Taq DNA polymerase (*Bangalore Genei*, Bangalore, India) and 50 ng betel vine DNA template in a 0.025 cm^3 reaction volume. The reaction mixes were overlaid with mineral oil prior to carrying out the amplification. The reaction was cycled 44 times at 94 °C for 1 min, 35 °C for 1.5 min and 72 °C for 1.5 min in a thermocycler (*Robocycler 40*, *Stratagene*, Heidelberg, Germany). The final extension cycle allowed an additional incubation for 5 min at 72 °C.

Agarose gel electrophoresis: Amplification products were separated by electrophoresis (at a constant current of 5 A) through 1.0 % agarose gels in 0.5 × TBE buffer according to Sambrook *et al.* (1989), visualized and imaged using gel documentation system (*Nighthawk*™, *pdi Inc.*, New York, USA) after staining with ethidium bromide.

Data Analysis: Data (fragment sizes of all the amplification products, estimated from the gel by comparison with standard molecular mass marker, λ DNA double digested with *Hind*III and *Eco*RI) were scored as discrete variables, using "1" to indicate presence and "0" to indicate absence of a band. From the band data, monomorphic and polymorphic bands were identified for each type of cultivars. A pair wise matrix of similarity between genotypes was determined for the cumulative RAPD (5 informative primers) data using an algorithm (Jaccard 1901) in the *RAPDistance* package (Armstrong *et al.* 1994). From the pair wise similarity data, the UPGMA phenogram was generated according to Sokal and Sneath (1963).

Results

The betel vine DNAs were tested in RAPD reactions in triplicate. Initially the pilot reactions including suitable controls and outgroup DNAs were carried out to determine the optimal primer, template and Mg^{2+} concentrations and to test the reproducibility of the reactions. The profiles were considered reproducible if at least two of the three DNA preparations revealed identical prominent bands after RAPD with a given primer (data not shown). Subsequently, the entire set of betel vine DNAs were tested with twenty-one decamer primers. Of these, 5 primers (F-01, F-03, F-04, F-13 and

Table 2. The decamer sequences used, which have resulted in consistent profiles, in RAPD reactions.

Primer name	Operon kit	Sequence (5' - 3')
OP-F01	F	ACGGATCCTG
OP-F03	F	CCTGATCACC
OP-F04	F	GGTGATCAGG
OP-F13	F	GGCTGCAGAA
OP-G18	G	GGCTCATGTG

Table 3. The pair-wise similarity indices from the RAPD band data

	B1	B2	B3	B5	B108	B104	B7	K212	K226	K228	K214	K218	K219	K238	K208	K6
B1	1.00															
B2	0.97	1.00														
B3	0.90	0.93	1.00													
B5	0.87	0.90	0.96	1.00												
B108	0.81	0.84	0.83	0.86	1.00											
B104	0.82	0.84	0.84	0.87	0.94	1.00										
B7	0.91	0.93	0.93	0.90	0.84	0.90	1.00									
K212	0.30	0.30	0.26	0.26	0.33	0.31	0.27	1.00								
K226	0.28	0.29	0.28	0.28	0.31	0.30	0.26	0.59	1.00							
K228	0.37	0.38	0.33	0.34	0.40	0.39	0.35	0.71	0.82	1.00						
K214	0.36	0.37	0.33	0.33	0.40	0.38	0.34	0.69	0.85	0.97	1.00					
K218	0.25	0.26	0.24	0.24	0.28	0.26	0.23	0.61	0.79	0.70	0.73	1.00				
K219	0.33	0.33	0.35	0.36	0.39	0.38	0.33	0.58	0.64	0.70	0.69	0.56	1.00			
K238	0.39	0.40	0.35	0.36	0.39	0.38	0.37	0.49	0.42	0.53	0.51	0.35	0.44	1.00		
K208	0.33	0.33	0.32	0.32	0.36	0.34	0.33	0.55	0.43	0.54	0.53	0.39	0.55	0.55	1.00	
K6	0.40	0.41	0.40	0.37	0.44	0.42	0.41	0.49	0.50	0.64	0.63	0.47	0.49	0.53	0.50	1.00

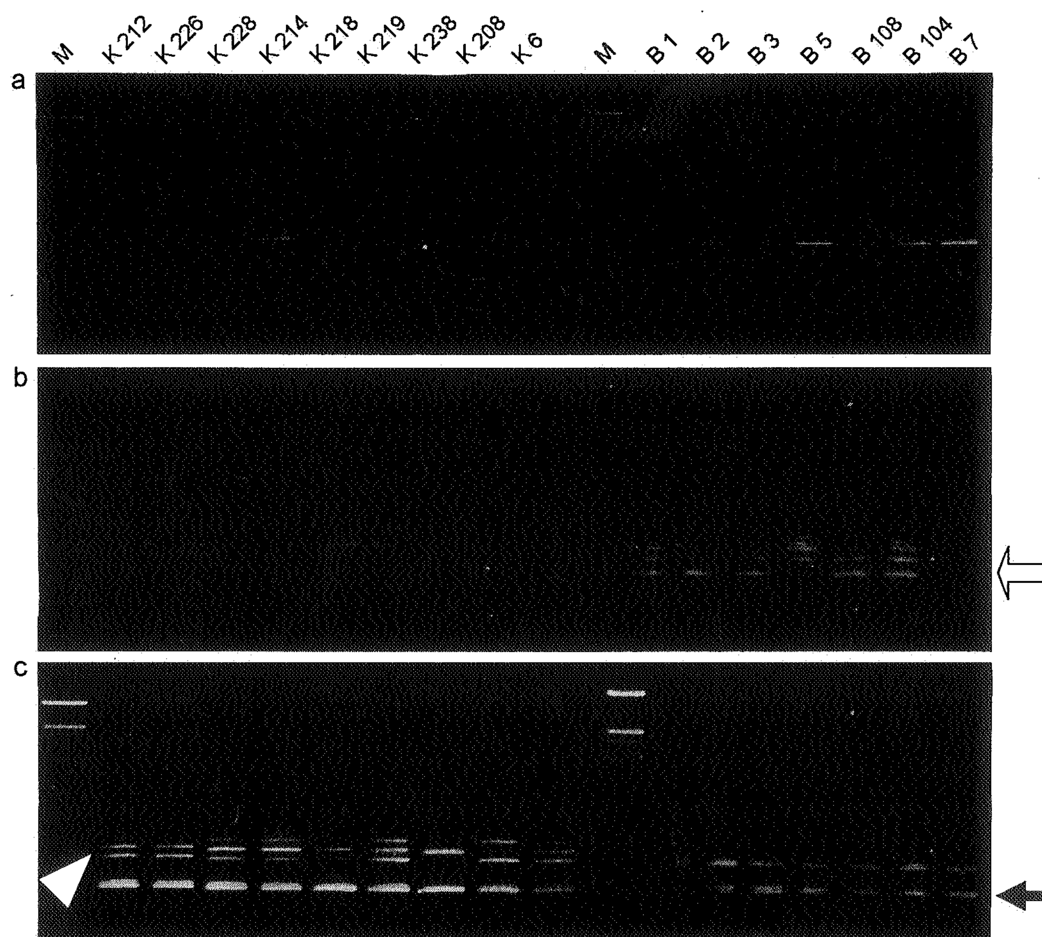

Fig. 1. RAPD agarose gel electrophoresis profiles of the betel vine cultivars using primers OP-G 18 (*a*), OP-F04 (*b*) and OP-F13 (*c*). *Lanes* indicated by M contain λ DNA double digested with *Hind*III and *Eco*RI as molecular mass marker. The cultivar numbers are according to Table 1 and indicate the template DNA from the appropriate cultivar. The *solid arrow* in *c* indicates one of the monomorphic bands present in all the betel vine cultivars used in the study. Similarly, the *white arrowhead* in *c* indicates the Kapoori specific band and the *large open arrow* in *b* indicates the Bangla specific band.

Table 4. The average pair-wise similarity indices for the RAPD band data in case of the betel vine cultivar-types (number of cultivars in parentheses). Mean probability $P = x^n$ for all n polymorphic bands in a cultivar are present in another random cultivar in the population where average similarity index is x (Bruford *et al.* 1992).

Type	Number of bands total	monomorphic	polymorphic	Polymorphic fraction of bands	Mean pairwise similarities	Mean probability
Bangla (7)	33	7	26	0.79	0.884	4.05×10^{-2}
Kapoori (9)	49	3	46	0.94	0.521	9.43×10^{-14}
Kapoori + Bangla	60	6	54	0.90	0.508	1.31×10^{-16}

G-18, Table 2) resulted in reproducible RAPD profiles in all the nine Kapoori and seven Bangla types (Fig. 1). The data from the five primers considered cumulatively resulted in a matrix of 60 bands for which presence or absence was scored in the cultivars. From this matrix, similarity indices were calculated amongst pairs of cultivars (Table 3). The Bangla cultivars B1 and B2 were found to have the highest similarity (SI = 0.97, Table 3) while the cultivars B1 and B108 exhibited the least similarity (SI = 0.81, Table 3). In the Kapoori cultivars, K228 and K214 were most similar (SI = 0.97, Table 3) while the cultivars K218 and K238 were the least similar (SI = 0.35, Table 3). Between Kapoori and Bangla the highest similarity (SI = 0.44, Table 3) was between B108 and K6 while the least similarity (SI = 0.23, Table 3) was between B7 and K218. The two types of betel vine cultivars, Bangla and Kapoori were clearly distinguished from each other in the UPGMA dendrogram (Fig. 2). The Kapoori cultivars were more heterogeneous (mean SI = 0.521, Table 4) as compared to Bangla cultivars (mean SI = 0.884, Table 4).

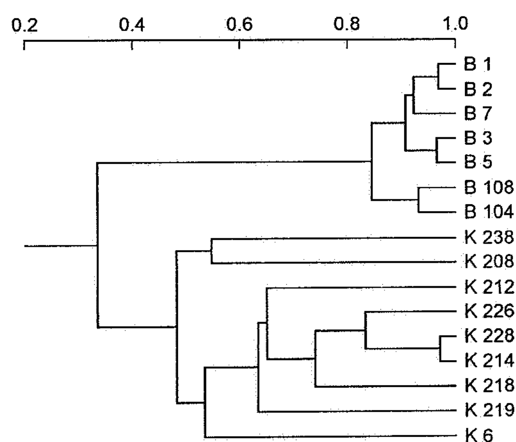

Fig. 2. Cluster analysis of cumulative RAPD data in the betel vine cultivars. The phenogram was generated by the UPGMA method. The cultivar numbers are indicated to the right of the phenogram. The scale on the top of the phenogram is a similarity scale based on the values in Table 3.

Discussion

Betel vine cultivar type Bangla comprises of nearly 35 cultivars, while the Kapoori type includes nearly 25 cultivars. The two cultivar-types are quite distinct from each other; however, the extent of variation among and between them is not easy to analyze due to the vegetative propagation. Under these conditions, RAPD technique could reveal within-cultivar type variation more efficiently.

In the present study, two Bangla cultivars, namely, Kalkattia Bangla and Calcutta Bangla have been selected to illustrate the nomenclature confusion. The two may well turn out to be identical if one considers that the former is named as a Bangla cultivar from Calcutta in West Bengal, India and the name is transliterated from Hindi language, while the latter is also a Bangla from Calcutta (English name of the cultivar). If these were identical cultivars representing only nomenclature confusion, then it was expected that the RAPD profiles with each and every primer tested would be identical. This is not so and the two cultivars have SI = 0.87. Thus these two cultivars are similar to each other but not identical. However, considering that the average similarity amongst the Bangla cultivars is 0.884, it indicates that the cultivars are highly similar to each other and are presumably propagated from an ancestral group that were also very similar to each other.

Alternatively, if one assumes that the cultivars are in fact identical, the small variations in RAPD profiles between them represent fixed somatic mutations. However, the present data on only seven Bangla cultivars and with only five primers may be inadequate for this alternative interpretation.

Our data on RAPD profiles has also revealed interesting relationship trends amongst the two types of betel vine cultivars. Out of the 60 bands analyzed, seven bands were specific to Bangla cultivars, three were specific to Kapoori cultivars and six bands were common to both Bangla and Kapoori cultivars. Similarly, Kapoori

cultivars were found to be more heterogeneous than Bangla cultivars (Table 4). We have also estimated the mean probability that all polymorphic RAPD bands present in any cultivar are also present by chance in any other random cultivar within the type. This value is very low in case of Kapoori type as well as between Kapoori and Bangla types (Table 4). This shows that the Kapoori type of cultivars is more heterogeneous. Similarly, it is also an extremely rare probability that all polymorphic bands in any Kapoori or Bangla cultivar will also be present in any random Bangla or Kapoori cultivar, respectively, by chance. Thus it is clear that the two types of cultivars differ from each other and that these have presumably evolved or domesticated from different ancestral types. Further, centuries of cultivation practices by vegetative propagation may have fixed the differences between these two cultivar-types in their populations. Some of the primers have resulted in bands specific to either Kapoori or Bangla or both together (Table 4). Such bands specific to Bangla or Kapoori cultivars can be developed further as SCAR markers for these types and may have a diagnostic value. Similarly the six bands monomorphic in both Bangla and Kapoori cultivars may be developed into SCAR markers specific to *Piper betle* in general.

RAPD technique has been employed to screen the germplasm in case of several higher plants. Most of these studies have been carried out in case of obligate to facultative cross-pollinated plants, and consequently, relatively higher estimates of genetic variability were obtained. In the case of tissue culture raised or micropropagated plants, RAPD technique has enabled the testing of fidelity of micro-propagation methods (Rani *et al.* 1995). In case of garlic, a seed sterile crop, RAPD analysis along with isozymes has allowed the infraspecific differentiation of the plants (Maass and Klaas 1995). Betel vine types are somewhat similar to the garlic plants as far as the lack of propagation through seed is concerned. However, the betel vines exhibit less variability in morphological characters as compared to that in the case of garlic. Betel vine cultivars like the natural populations of *Crotalaria longipes*, have also shown low genetic polymorphism (Jayanthi and Mandal 2001). The RAPD profiles, however, could reveal relative variability within as well as between the two types of betel vine cultivars. Clearly there is scope for large-scale application of RAPD for analysis of such obligate vegetatively propagated plants.

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