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EVALUATION OF GENETIC VARIABILITY AMONG *BIDI* TOBACCO VARIETIES BY RAPD

K.B.J. PRASSANNA RANI AND K. SIVA RAJU

Central Tobacco Reserach Institute, Rajahmundry – 533 105

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Randomly amplified polymorphic DNA (RAPD) markers were used to study the genetic variability among the *bidi* tobacco varieties grown in India. A total of 47 amplified fragments were produced by 13 primers in the cultivars, of which 24 were polymorphic with 51.06% of polymorphism. The primer OPP1 amplified 500 bp fragment specific to the variety Bhagyasree, whereas the primer OPB1 produced a fragment of 200 bp specific to the variety GT-9. The genetic similarity among the varieties varied between 64 and 95%. The maximum genetic similarity (95%) was observed between varieties NPN-190 and Bhavyasree, whereas it was minimum (64%) between the varieties GT-9 and GT-5. On the basis of Unweighted pair group method on arithmetic averages (UPGMA) method of clustering analysis, the 10 varieties separated into two main clusters and the variety GT-9 independently linked to the main cluster. Based on RAPD markers, *bidi* tobacco cultivars were separated into two main clusters and the sub-clustering pattern was mainly based on parentage of varieties and also based on the region and it revealed close genetic diversity in *bidi* tobacco. The specific markers identified and amplification profiles of the varieties generated in the present study will be helpful in identifying the varieties.

INTRODUCTION

Assessment of genetic diversity is critical for any successful breeding programme. Usually, breeders employ morphological markers for genetic diversity estimation. *Bidi* tobacco (*Nicotiana tabacum* L.) belongs to the genus *Nicotiana* of the family Solanaceae. India is the only country where several types of tobacco, *viz.*, Flue-Cured Virginia (FCV), chewing, burley, *natu*, cigar filler, cigar wrapper, cheroot, *hookah*, HDBRG and *bidi* are grown under different agro-climatic conditions. The qualities of tobacco grown in different locations differ considerably depending on the genotype, climatic conditions and cultural practices adopted. Among the different types of tobacco grown in India, *bidi* tobacco ranks second in area (25%) but stands first in production (38%). From the productivity point of view, Gujarat ranks first among the tobacco growing states and more than 80% of *bidi* tobacco grown in the country is produced in Gujarat. In India, cultivation of *bidi* tobacco is concentrated in Charotar area of Gujarat, Nipani in Karnataka, Kolhapur and Sangli districts in Maharashtra, Danlewada district of Chhattisgarh and in Kurnool district of Andhra Pradesh. *Bidi* tobacco fetches sizeable income to the farmers, traders and government and provides employment to millions of people involved in its cultivation, curing, grading, processing and marketing.

Identification of crops has become increasingly important with the awareness about Plant Breeders Rights and cultivar registration. Tobacco cultivars are currently distinguished by morpho-physio-chemical characters but this method is slow, unrealistic and phenotypic identifications based on morphological traits is subjected to environmental variations (Nielsen 1985). Moreover low level of genetic diversity within and among cultivated tobacco types was reported (Ren and Timko 2001). Hence, cultivars that are closely related cannot be readily distinguished by morphological indices.

Information regarding DNA polymorphism is more elaborative than phenotypic level to properly explain the genetic diversity and to findout more diverse genotypes for crossing programme. The PCR based molecular markers have been used for analysis of genetic variations in natural populations. One such technique using arbitrary primers, namely RAPD is a convenient, easy to perform and requires no information about the DNA sequence to be amplified and provide

rapid assessment of the differences in the genetic composition of the related individuals. RAPD has been used as molecular markers in cultivar characterization for *Pyrus cummunis* L. (Shivapriya and Shailaja, 2006), Musa (Jain *et al.*, 2007), *Cuscuta reflexa* (Khan *et al.*, 2010) and *Ruta graveolens* (Khan and Abdin, 2011). DNA fingerprinting in different tobacco types by RAPD and AFLP markers was reported (Ren and Timko 2001, Siva Raju *et al.*, 2008). There was no systematic information on the existing diversity pattern in *bidi* tobacco varieties, which is crucial to define future breeding strategy in this important crop. This paper reports the genetic diversity based on RAPD analysis among 10 *bidi* tobacco varieties grown in India.

MATERIALS AND METHODS

Plant material and DNA isolation

Ten genotypes of *bidi* tobacco (Table 1) comprising released varieties cultivated in Gujarat, Karnataka, Maharastra and Andhra Pradesh were subjected to RAPD analysis. This collection represents different commercial *bidi* tobacco varieties defined by their morphological and biochemical characteristics. Thirty-day-old seedlings of each variety were collected, frozen in liquid nitrogen, and stored at -80 C until used for DNA extraction. DNA was extracted from pulverized frozen seedlings as per the modified protocol by Doyle and Doyle (1990). The DNA concentration was estimated by comparing with known concentration of lambda DNA as standard in agarose gel electrophoresis (0.8% gel containing 0.5 µg/ml ethidium bromide).

Polymerase chain reaction (PCR)

A total of 20 decamer primers (Operon Technologies Inc, Alameda, CA, USA) were screened for RAPD analysis. Of these, 13 primers (OPP-1, OPP-3, OPP-4, OPP-9, OPB-14, OPP-10, OPP-12, OPAB-18, OPB-1, OPL-2, OPL-14, OPM-10 and OPM12) were selected for analysis based on repeatability. The PCR amplification conditions were as described by Williams *et al.* (1990) with minor modifications. Amplifications were carried out in a 25 ml reaction mixture containing 25 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 20 ng of each primer. Amplification was carried out in a DNA thermal cycler (PTC-100, Peltier thermal cycler, M.J. Biotech, USA) programmed to run the following thermal profile: initial denaturation at 94°C for 5 min, 40 cycles of 94°C x 1 min, 37°C x 1 min, 72°C x 2 min, followed by final extension at 72°C for 10 min. Amplification products were resolved on 1.4% agarose gel at 50 V and gels were documented in gel documentation system (Gene genius, Syngene bio-imaging system, U.K). The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Data analysis

All the genotypes were scored for presence or absence of the RAPD bands. Only intense

Table 1: Characters of the varieties used in the present study

S.no.	Variety	Parentage	Intended use	Growing state
1	GT-9	ABD 64 line	*Bidi*	Gujarat
2	GT-7	930-47 x Anand 2	*Bidi*	Gujarat
3	GT-5	GT-4 x 108-9- 101	*Bidi*	Gujarat
4	GT-4	70-6-6 x Anand 119	*Bidi*	Gujarat
5	Anand-2	Sokhadu x 88-47	*Bidi*	Gujarat, Karnataka, Maharashtra, Andhra Pradesh
6	Anand-119	Local selection	*Bidi*	Gujarat, Karnataka, Maharashtra, Andhra Pradesh
7	GTH-1	CMS bidi hybrid	*Bidi*	Gujarat
8	NPN-190	320 X (169-119 x Olar) -121-2-1	*Bidi*	Karnataka, Maharashtra
9	Bhavyasree	NPN190 x PL-5	*Bidi*	Karnataka
10	Bhagyasree	NPN 23 x PL-5	*Bidi*	Karnataka

bands and those that were repeatedly amplified were scored visually. The data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The Excel file containing the binary data was imported into NT Edit of NTSYS-pc software version 2.02 (Rohlf, 1998). The 0/1 matrix was used to calculate similarity as DICE coefficient using SIMQUAL subroutine in Similarity routine. The resultant similarity matrix was employed to construct dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny.

RESULTS AND DISCUSSION

Extent of genetic variation in *bidi* tobacco

RAPD markers were employed to characterize *bidi* tobacco (*Nicotiana tabacum* L.) varieties grown in different states of India. Out of 20 primers used in RAPD, reproducible patterns were obtained with 13 primers, which generated a total of 47 highly reproducible bands in the size ranged from 0.2 to 2.6 kb. The total number of fragments amplified per primer ranged from 3 to 7 with an average of 3.38 (Table 2). Eleven (84.6%) primers were found to be polymorphic while the primers OPP3 and OPAB18 were monomorphic. The level of polymorphism detected with different primers ranged from 0 to 100%. Out of all the 47 amplified fragments, 24 (51.06%) were polymorphic. The observed level of polymorphism in *bidi* tobacco was very much higher than that reported by Del Piano *et al.* (2000) and Yang *et al.* (2006) in tobacco but it was nearer to that report by Siva Raju *et al.* (2008), where 59.4% polymorphism has been documented in 10 types of tobacco by RAPD, it may be due to different tobacco types. Besides, the primers used in the present study being different in sequence, possibly amplified different genome regions. A total of 23 bands were common to all the cultivars indicating the close evolutionary relationship in *bidi* tobacco. The primer OPAB 18 produced fragments which are common to all the cultivars and size of the amplified fragments varied between 500 and 2000 bp. The primer OPP1 amplified 500 bp fragment specific to the cultivar Bhagyasree (Fig. 1) whereas the primer OPB1 produced a fragment of 200 bp specific to the variety GT-9. The primers namely OPP 9, OPB14, OPP 12, OPL 2, OPL14 and OPM20 (Table 2) used in this study were found to be highly polymorphic. Therefore, these primers could be employed in the future RAPD based diversity analysis of *bidi* tobacco lines and germplasm.

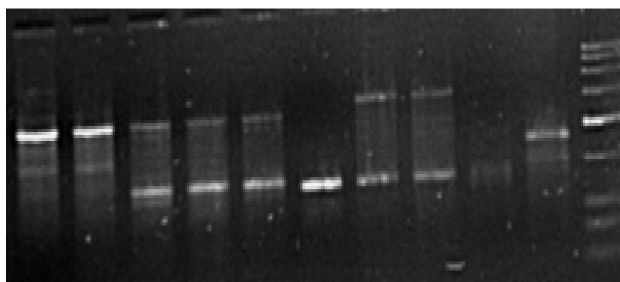

Fig. 1: RAPD amplification by OPP1. 1. GT-9, 2. GT-7, 3. GTH-1, 4. GT-4, 5. Anand-2, 6. Anand-119, 7. GT-5, 8. NPN-190, 9. Bhavyasree and 10. Bhagyasree. M-Low range DNA ladder

Genetic relationship among the genotypes

The genetic relationship among *bidi* tobacco genotypes was established by RAPD markers. From Jaccard's coefficient of similarity for all varieties, the pair-wise similarity comparisons ranged from 0.64 to 0.95 with an average of 0.67.

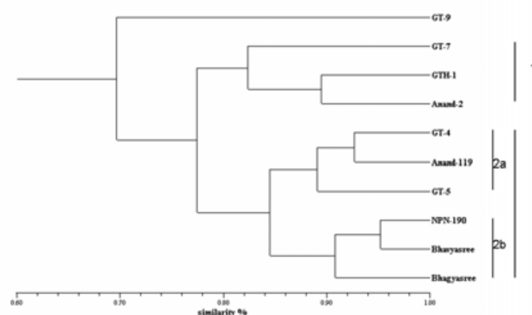

Fig. 2: Dendrogram constructed using an UPGMA and SAHN algorithm from the Jaccard's similarity matrix data from all the RAPD marker profiles consisting of amplified fragments, revealing genetic relationship among the varieties

Table 2: Data on RAPD amplification

Primer	Sequence of primer	Total number of bands	Number of polymorphic bands	Per cent of polymorphism	Specific bands
OPP-1	GTAGCACTCC	4	2	50	500 bp- specific to Bhagyasree
OPP-3	CTGATACGCC	3	0	0	-
OPP-4	GTGTCTCAGG	3	1	33	-
OPP-9	GTGGTCCGCA	3	2	66	-
OPB-1	TCCCGCCTAC	4	2	50	2000bp-specific to GT- 9
OPB-14	AAGGGCGAGT	5	5	100	-
OPP-10	AGTTCGCTCC	3	0	0	-
OPP-12	TCCGCTCTGG	3	2	66	-
OPAB-18	CTGGCGTGTC	3	0	0	-
OPL-2	TGGGCGTCAA	3	2	66	-
OPL-14	GTGACAGGCT	3	2	66	-
OPM-10	TCTGGCGCAC	7	3	43	-
OPM-20	AGTCTTGGGC	3	3	100	-

The varieties GT-9 and GT-5 showed minimum genetic similarity (64%) whereas the varieties NPN-190 and Bhavyasree showed maximum genetic similarity (95%). Similarity coefficient matrices were used to generate a dendrogram based on UPGMA method of clustering analysis.

In UPGMA method of clustering analysis, the 10 varieties separated into two main clusters and the variety GT-9 independently linked to the main cluster (Fig.2). The cluster 1 was formed by 3 varieties GT-7, GTH-1 and Anand 2. The variety Anand 2 was one of the parents to variety GT-7 and showed 79% of genetic similarity. The variety GTH-1 was a male cytoplasmic sterile hybrid and showed 89% of genetic similarity with the variety Anand 2 and 74% with GT-7. The second cluster was formed by 6 varieties with two sub-clusters. The varieties grown in Gujarat state formed sub-cluster 2a and the varieties grown in Karnataka formed sub-cluster 2b. The sub-cluster 1a was formed by GT-4, Anand 119 and GT-5. The variety Anand 119 was one of the parents to the variety GT-4 whereas GT-4 was one of the parents to the variety GT-5. The sub-cluster 2b was formed by the varieties NPN190, Bhavyasree and Bhagyasree. The variety NPN 190 was one of the parents to Bhavyasree and showed 95% genetic similarity. The variety Bhagyasree showed 90% genetic similarity with the variety Bhavyasree and this close genetic similarity may be due to the fact that both these varieties had a line PL-5 as one of the parents. Thus, the sub-clustering formation was based on parentage.

The genetic similarity among the cultivars was very high indicating that there was no significant gene flow among the cultivars, possibly due to the preferential use of one or two varieties as parents in the breeding programme, because of prepotency of these varieties to produce desirable qualitative traits and their adaptability to particular agro-climatic conditions. The main clusters and the sub-clustering pattern was mainly based on parentage of varieties and also based on the region *i.e.* the varieties grown in Karnataka state was separated from those grown in Gujarat state. The specific markers identified in the present study will be helpful in identifying the varieties.

Thus, RAPD markers were efficient in detecting polymorphism among the *bidi* tobacco cultivars showing their utility in validation / characterization of *bidi* tobacco cultivars, even in case of very closely related varieties. The RAPD analysis carried out in this study revealed close genetic diversity in *bidi* tobacco. Therefore, rational use of genetic resources in future breeding programmes is warranted to generate

a higher level of diversity in the newly released cultivars.

REFERENCES

Atienzer, F., A. Evenden, A. Jha, D. Savva and M. Depledge. 2009. Optimized RAPD analysis generates high quality genomic DNA profiles at high annealing temperatures. **Biotechniques** 28: 52-4.

Del Piano, L., M. Abet, C. Sorrenlino, F. Acanfora, E. Cozzolino, F. Acanfora, E. Cozzolino and A. DiMuro. 2000. Genetic variability in *Nicotiana tabacum* and *Nicotiana* species as revealed by RAPD procedure. **Beitrege Zur. Tab. Int.** 19: 1-15.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. **Focus** 12: 13-5.

Jain, P.K., M.L. Saini, H. Pathak, and V.K. Gupta, 2007. Analysis of genetic variation in different Banana (*Musa* species) variety using random amplified polymorphic DNAs (RAPDs). **African J. Biotech**. 6: 1987-9.

Khan, S., K.J. Mirza and M.Z. Abdin. 2011. DNA fingerprinting for the authentication of *Ruta graveolens*. **African J. Biotech**. 10: 8709-15.

Khan, S., K.J. Mirza and M.Z. Abdin. 2010. Development of RAPD markers for authentication of medicinal plant *Cuscuta reflexa*. **EurAsian J. Biosciences**. 4: 1-7.

Nielsen, G. 1985. The use of isozymes as a probe to identify and label plant varieties and cultivars. In: *Isozymes: Current Topics in Biological and Medical Research* (M.C. Rattazzi, J.G. Scandalios and G.S. Whitt, Eds.), Vol. 12. Alan R. Liss, New York, pp. 1-32.

Ren, N. and M.P. Timko. 2001. AFLP analysis of genetic polymorphism and evolutionary relationships among cultivated and wild *Nicotiana* species. **Genome** 44: 559-71.

Rohlf, F.J. 1998. NTSYS-pc: Numerical taxonomy and multivariate analysis system. Version 2.02, Exter Software, Setauket, New York.

Siva Raju, K., M.S. Madhav, R.K. Sharma, T.G.K. Murthy, N.K. Singh, K.C. Bansal, K.R. Koundal and T. Mohapatra. 2008. Molecular diversity in Indian tobacco types as revealed by Randomly Amplified DNA polymorphism. **J. Plant Biochem. Biotech.** 17: 51-6.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Res.** 18: 6531-5.

Yang, B.C., B.G. Xiao, X.J. Chen and C.H. Shi. 2006. Assessing the genetic diversity of tobacco germplasm using intersimple sequence repeat and inter-retrotransposon amplification polymorphism markers. **Ann. Appl. Biol.** 150: 393-401.