RESEARCH NOTE

Genetic diversity in Indian FCV and burley tobacco cultivars

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Introduction

Tobacco is an important commercial crop of India. Though it has never been found in wild state, cultivated tobacco is highly polymorphic with wide range of morphological types and diverse uses. Most of the commercial tobaccos produced in the world are *Nicotiana tabacum*. The only other species used on a limited commercial scale is *N. rustica* (Goodspeed 1954). India is the only country where many different types of tobacco, viz., flue-cured Virginia (FCV), burley, natu, cigar filler, cigar wrapper, cheroot, hookah, bidi and chewing are grown under different agro-climatic conditions. Among them FCV and burley are exportable tobacco types and are mainly used in cigarette manufacturing. Indian FCV tobacco is famous in the world market for its filling quality in cigarettes, and burley tobacco for cigarette blending. FCV tobacco is mainly grown in Andhra Pradesh (AP) and Karnataka states of India, and burley in AP only. The kind of tobacco grown in an area differs considerably with respect to climatic conditions, nutrient supply and use of the end product. Hence, breeding of tobacco varieties is location-specific, right from the choice of parents to the final stages of evaluation and selection. This approach has resulted in the development of a large number of location specific high yielding tobacco varieties.

Varietal diversification is apparent among commercial cultivars of FCV and burley tobaccos grown in India. Assessment of their genetic diversity is of importance for long-term tobacco improvement and reduction of vulnerability of the crop. Prior to the availability of DNA-based markers, most genetic diversity studies in various crops were carried out using morphological markers. Molecular markers have become useful tools to provide a relatively unbiased method of quantifying genetic diversity in plants (Clegg 1990). Genetic diversity has been studied in many crops using markers like restricted fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites (Aggarwal *et al*. 1999; Angiolillo *et al*. 1999). RAPD is a PCR based method being preferred in crop plants because of the relative ease with which PCR assays can be carried out compared to RFLP. Besides, prior knowledge about the genome is also not a prerequisite, which makes RAPD a common method for such studies in different crops (Nair *et al*. 2002). In tobacco, the RAPD technique has been mainly used to identify markers linked to genes for resistance to pathogens (Bai *et al*. 1995; Yi *et al*. 1998). Previously, a few studies have assessed the genetic diversity in tobacco cultivars using RAPD and found a low degree of polymorphism (Xu *et al*. 1998; Del Piano *et al*. 2000; Zhang *et al*. 2005).

Although a large number of tobacco varieties belonging to different commercial types have been developed in India, complete information is not available on their genetic base and diversity. This paper reports the results of a study on the genetic diversity based on RAPD among 10 tobacco (eight FCV and two burley) varieties that are currently grown in different tobacco growing areas of India.

Material and methods

Eight FCV tobacco varieties (Hema, VT 1158, Hemadri, Kanthi, CM 12, Kanchan, Bhavya and FCV Special) and two burley cultivars (Banket-A1 and BSRB-II) were used to study RAPD profiles. All these lines are high yielding ruling tobacco cultivars. Hema and VT 1158 (resistant to tobacco mosaic virus) are popular FCV cultivars of black soil areas of AP. Hemadri is recently released for cultivation in the central black soil areas of AP and Kanthi for southern black soil and light soil areas. CM 12 is one of the recommended varieties for northern light soils (NLS) areas of AP. Kanchan is a semi-flavourful tobacco cultivar grown in both northern light soil (NLS) and Karnataka light soil (KLS) areas. Bhavya and FCV Special are the recommended varieties for KLS area.

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Banket-A1 is a burley variety grown in agency tobacco areas of AP and BSRB-II is a pipeline burley line.

DNA extraction and RAPD assay

Tobacco seedlings used for DNA extraction were raised on moist filter paper in a tissue culture room. DNA was extracted from 30-day-old seedlings of each variety following Doyle and Doyle (1990) with minor modifications, and quantified on 0.8% agarose gels. The stock DNA was diluted to make a working solution of 30 ng/μl for PCR analysis.

The isolated DNA was used for PCR amplification as described by Williams *et al*. (1990) with minor modifications. Amplifications were carried out in a 25 μl reaction mixture containing 30 ng template DNA, 0.5 units of *Taq* polymerase, 0.2 mM dNTPs and 30 ng of each primer using PCR Sprint (Thermo Hybaid, Franklin, USA). Twenty four random primers (Operon Technologies, Alameda, USA) viz. OPB-1, OPB-14, OPD-1, OPD-5, OPD-9, OPD-12, OPD-20, OPE-1, OPE-3, OPE-6, OPE-11, OPF-8, OPF-16, OPH-1, OPH-5, OPL-9, OPM-2, OPM-6, OPM-10, OPM-13, OPN-7, OPN-8, OPN-15 and OPN-20 were used for the amplification. Only primers that had been earlier found to be polymorphic among tobacco cultivars (Xu *et al*. 1998) were used in this study. PCR cycles consisted of one cycle of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 37°C for 1 min and 72°C for 2 min, with a final primer extension cycle of 7 min at 72°C. Amplified PCR products were separated on 0.8% (w/v) agarose gels having 0.5 mg/ml ethedium bromide in 1× Tris-acetate-EDTA (TAE) buffer. Images of gels were captured using Gel Doc-2000 (Biorad, Sydney, Australia).

Data analysis

The binary RAPD data were recorded depending on the presence or absence of the bands obtained after the RAPD analysis. Amplified bands were scored as present (1) or absent (0). The genetic variation among the tobacco lines were analysed using the NTSYS-pc software version 2.02 (Rohlf 1998) using the RAPD binary data. Using the software, Jaccard's (1908) similarity coefficients among 10 cultivars were calculated and a dendrogram depicting the genetic relationship among the varieties was prepared based on UPGMA and SAHN clustering. Percentage of polymorphic bands was defined as ratio of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer, expressed in percentage.

Results and discussion

In the present study, 24 random primers were tested for their ability to produce polymorphic bands in 10 tobacco lines. The RAPD exhibited both high-intensity bands and low-intensity bands. Only those bands that showed differences in their migration on agarose gel were considered in this study, and differences in the intensity of fluorescence were ignored.

Among the 24 tested primers, OPC-19 did not show any amplification and hence these bands were scored for only 23 primers (table 1). A total of 105 bands were scored from the amplifications with 23 primers of DNA from 10 tobacco cultivars, with an average of 4.6 bands scored per primer. The size of the amplified products ranged from 220–2860 bp in different primers. 74% of the bands were found to be polymorphic. Total number of bands produced by different primers ranged from one (OPD-5) to ten (OPN-8), while the polymorphic bands ranged from one (OPD-5, OPH-1, OPM-2 and OPM-10) to nine (OPE-6 and OPN-8). The per cent polymorphic bands ranged from 0% (OPN-20) to 100%. Ten primers produced 100% polymorphic bands. By comparing with the previous studies, Del Piano *et al*. (2000) reported complete monomorphic pattern in 36 tobacco lines belonging to different tobacco types, using RAPD primers, whereas Zhang *et al*. (2005) obtained 63% polymorphic bands with 24 tobacco cultivars using 17 RAPD primers.

The variation in banding pattern in the present study indicated a relatively higher degree of polymorphism among the cultivars studied. High degree of polymorphism was also observed among the *Nicotiana* species using two RAPD primers (Del Piano *et al*. 2000) and AFLP markers (Ren and Timko 2001), whereas Del Piano *et al*. (2000) reported very low level of polymorphism in 12 lines of tobacco using RAPD.

Values of Jaccard's similarity and coefficients among different varieties ranged between 0.798 and 0.571 with an average of 0.668, indicating close relationship among the cultivars studied (table 2). Genetic similarity among FCV cultivars found to be higher (0.70) compared to burley (0.65). Low similarity among burley cultivars may be due to the interspecific origin of BSRB-II. FCV cultivars of both states recorded higher similarities within each group (0.72) and lower between the groups (0.683), while the genetic similarities between FCV and burley groups were found to be still lower (0.545). Higher degree of genetic similarity was observed between Hemadri and CM-12 (0.798), and also in Hemadri and Kanthi (0.780). Bhavya recorded minimum genetic similarity values with Banket-A1 (0.571) and BSRB-II (0.575). Lowest similarities of Karnataka origin FCV tobacco cultivar, Bhavya, and burley cultivars Banket-A1 and BSRB-II of AP are expected, in view of their differences in type and geographical adaptation. Zhang *et al*. (2005) assessed the polymorphism, similarities and relationships among 24 *N. tabacum* cultivars with RAPD analyses and found higher similarities between cultivars.

Dendrogram was constructed using UPGMA cluster analysis depicting genetic relationships among 10 tobacco lines showed only two major clusters though there are six subclusters (figure 1). As expected, all FCV cultivars: Hema, VT-1158, Hemadri, Kanthi, CM-12, Kanchan, Bhavya and FCV Special, were grouped into a cluster and burley varieties Banket-A1 and BSRB-II into other. In the FCV cluster,

Table 1. Number of bands scored in different tobacco lines with different RAPD primers.

	Primer	Sequence	Total no. of bands	Polymorphic bands	%Polymorphic bands
1	OPB-01	5′ GTTTCGCTCC 3′	3	3	100
2	OPB-14	5′ TCCGCTCTGG 3′	3	3	100
3	OPD-01	5′ ACCGCGAAGG 3′	2	2	100
4	OPD-05	5′ TGAGCGGACA 3′	1	1	100
5	OPD-09	5′ CTCTGGAGAC 3′	5	5	100
6	OPD-12	5′ CACCGTATCC 3′	2	2	100
7	OPD-20	5′ ACCCCGTCAC 3′	2	2	100
8	OPE-01	5′ CCCAAGGTCC 3′	3	2	67
9	OPE-03	5′ CCAGATGCAC 3′	5	5	100
10	OPE-06	5′ AAGACCCCTC 3′	9	9	100
11	OPE-11	5′ GAGTCTCAGG 3′	6	2	33
12	OPF-08	5′ GGGATATCGG 3′	5	3	60
13	OPH-01	5′ GGTCGGAGAA 3′	3	1	33
14	OPH-05	5′ AGTCGTCCCC 3′	6	5	83
15	OPL-09	5′ TGCGAGAGTC 3′	5	2	40
16	OPM-02	5′ ACAACGCCTC 3′	2	1	50
17	OPM-06	5′ CTGGGCAACT 3′	8	6	75
18	OPM-10	5′ TCTGGCGCAC 3′	2	1	50
19	OPM-13	5′ GGTGGTCAAG 3′	7	6	86
20	OPN-07	5′ CAGCCCAGAG 3′	7	4	57
21	OPN-08	5′ ACCTCAGCTC 3′	10	9	90
22	OPN-15	5′ CAGCGACTGT 3′	4	4	100
23	OPN-20	5′ GGTGCTCCGT 3′	5	0	0
	Total		105	78	74

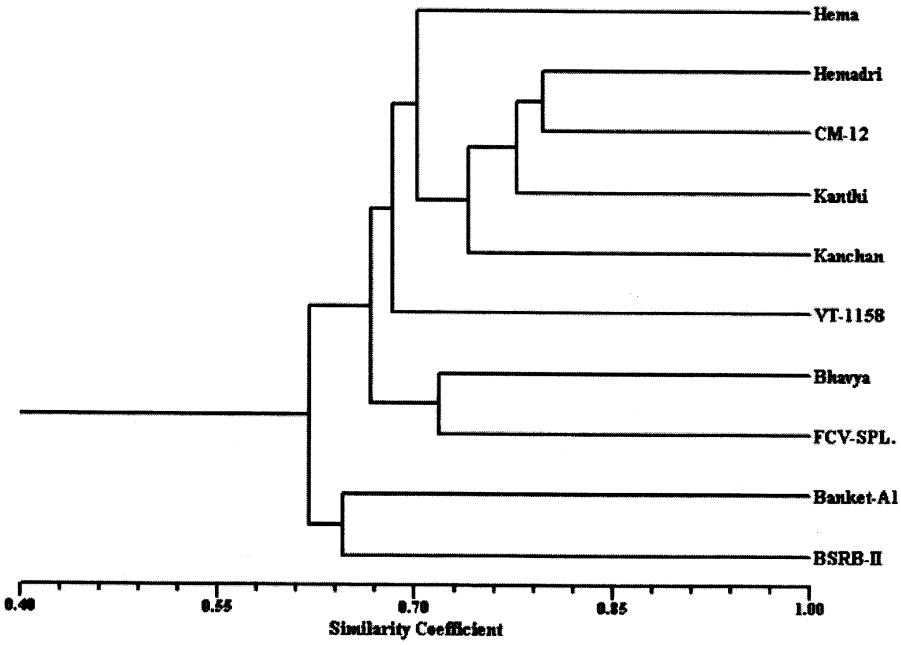

Figure 1. Dendrogram of the tobacco cultivars used in this study.

CM 12 and Hemadri formed one subcluster, to which Kanthi, Kanchan, Hema and VT-1158 were added in sequence. All these are the cultivars adapted to AP state. Bhavya and FCV Special, which are exclusively grown in Karnataka state, formed another subcluster. Though the variety Kanchan is grown in both the states, it clustered with the AP cultivars. Thus, the dendrogram indicates a clear pattern of division among the FCV varieties based on geographic origin, as also

Table 2. Genetic similarity coefficients among tobacco lines based on the RAPD data.

Tobacco varieties	Hema	VT-1158	Hemadri	Kanthi	CM-12	Kanchan	Bhavya	FCV-Special	Banket A-1	BSRB-II
Hema	1.000									
VT-1158	0.648	1.000								
Hemadri	0.678	0.732	1.000							
Kanthi	0.755	0.696	0.780	1.000						
CM-12	0.674	0.667	0.798	0.774	1.000					
Kanchan	0.699	0.674	0.722	0.742	0.756	1.000				
Bhavya	0.621	0.650	0.624	0.634	0.699	0.706	1.000			
FCV Special	0.690	0.605	0.655	0.681	0.690	0.736	0.718	1.000		
Banket A-1	0.593	0.639	0.671	0.625	0.648	0.693	0.571	0.586	1.000	
BSRB-II	0.635	0.605	0.619	0.630	0.598	0.625	0.575	0.590	0.646	1.000

reported in other crops (Spooner *et al*. 1996; Paul *et al*. 1997) and tobacco (Zhang *et al*. 2005).

The analysis clearly distinguished burley and FCV cultivars. Such studies can be used to study genetic differences of varieties for their identification. RFLP and RAPD markers most often arise from single or low copy sequence regions of the genome. Approximately 77% of the total nuclear DNA in cultivated tobacco is composed of repetitive sequences (Narayan 1987), the majority of which appears to have been conserved during the process of molecular evolution in this genus (Volkov 1999). Nonrepetitive DNA, that includes a majority of structural genes, makes up only a very small fraction of total tobacco genome, but should be responsible for most of the variations in morphology, growth characteristics, biochemical composition and quality traits found among varieties. Therefore, it might be predicted that RAPD may be effective in analysing polymorphism at the subspecies level in genus *Nicotiana*. In the present study RAPD markers provided sufficient resolution to distinguish closely related cultivars.

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