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SSR Based Genetic Diversity In Indian Flue-Cured Virginia Tobacco (*Nicotiana tabacum* L.) Cultivars

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Abstract

The genetic diversity among 24 Flue-cured Virginia (FCV) tobacco cultivars was investigated using 20 SSR primers. Distinct polymorphism was noticed among closely related cultivars studied indicating the robust nature of microsatellites in revealing polymorphism. A total of 78 amplicons were produced of which 48% were polymorphic among the varieties. The pairwise similarity among the varieties ranged from 0.54 to 0.91, indicated a broad genetic base of the tested genotypes which could be due to use of divergent parents including exotic introductions and local selections in breeding for different quality and physiological traits. Varieties developed indigenously through different breeding methods were clustered together whereas exotic introductions and their derivatives were grouped together separately. Genetic relationship among the FCV tobacco varieties based on polymorphic DNA profile largely corresponded with parentage. The SSR based analysis in the present study suggested that the Indian tobacco breeding has been successful in developing varieties with a relative broader genetic base.

Keywords: Indian FCV tobacco, Genetic diversity, SSR markers

Introduction

Tobacco (*Nicotiana tabacum* L) belongs to genus *Nicotiana* of the family Solanaceae. In the genus *Nicotiana*, out of 68 recognized species [1], only two species namely, *tabacum* and *rustica*, which are natural amphidiploids (*2n=48*), are grown commercially in the world. India is the only country where 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. The tobacco types are grown in India are broadly classified as Flue-cured Virginia (FCV) and non-FCV types. The qualities of FCV tobacco grown at different locations differ considerably depending on the soil, climatic conditions, water and nutrient supply. Hence breeding and selection of tobacco varieties is specific to the location, right from the choice of parents to the final stages of evaluation and selection.

Commercial cultivation of FCV tobacco in India dates back to 1929 and is highly exportable. Prior to 1970, tobacco cultivation in India was confined to a few introductions such as Harrision Special, Chatam, *etc*. During 1970, emphasis was given to pure line selection from the local landraces, which led to the development of popular varieties such as CTRI Special and Hema. During this period, hybridization involving the introductions and local selections was also carried out leading to the release of several high yielding varieties. After 1980, emphasis shifted towards development of varieties for biotic and abiotic stress tolerance through recombination breeding, back cross method and induced mutations. This approach resulted in the development of many stress tolerant varieties including Jayasri MR (Tobacco mosaic virus resistant) and the high yielding FCV variety Swarna (1983), possessing resistance to powdery mildew disease. The variety Kanchan, considered as a semi-flavorful variety was released in 1996 for the NLS area [2] and Siri, a high yielding and superior quality FCV variety was released in 2006. In addition to this, Indian tobacco is considered to contain lower levels of carcinogenic substances like tobacco specific nitrosoamines (TSNA) (0.42 to 1.44 ppm) as compared to tobacco from other countries [3].

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. Low level of genetic diversity within and among cultivated tobacco types was reported [4]. Cultivars that are closely related or with low genetic variability cannot be readily distinguished by morphological indices. Genetic and phenotypic characterization of crops has become increasingly important with commercial interest in Plant Breeders Rights and cultivar registration.

Molecular genetic markers have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants. PCR based markers like Randomly Amplified Polymorphic DNA (RAPDs), Simple Sequence Repeats (SSRs) and Amplified Fragment Length Polymorphism (AFLP) have an apparent advantage as cultivar descriptors in that they are unaffected by environmental or physiological factors [5]. Among different molecular markers, SSRs are more abundant, ubiquitous in presence, hyper variable in nature and have high polymorphic information content. These markers have been used for genotype identification in many crop plants such as brinjal [6], rice genotypes [7] and Jute [8]. Thus these markers are ideal for discriminating individuals and for parentage determinations.

RAPD has been used mainly in tobacco to identify markers linked to genes for resistance to pathogens [9] and genetic diversity [10,11]. AFLP markers were also employed with a few sets of varieties and species to know inter and intra specific variations in the genus *Nicotiana* [4]. Recently, with the advent of high-density SSR

maps for tobacco it is possible to estimate genetic variation with a large number of SSR markers that are well distributed across the tobacco genome [12]. SSRs as reproductively, co-dominant, wide genome coverage and multi allelic markers has been successfully employed to reveal the genetic variation of chewing tobacco genotypes [13]. Darvishzadeh *et al*. [14] studied the genetic diversity among Iranian water pipe tobacco varieties using SSR markers. Systematic effort has not been made to understand the existing diversity pattern in Indian FCV tobacco varieties, which is crucial to define future breeding strategy in this important exportable crop. This paper reports the genetic diversity based on SSR analysis among 24 released Indian FCV tobacco varieties which are currently cultivated in different agro-climatic zones of India.

Materials and methods

Plant material and DNA isolation

Seeds of 24 FCV tobacco varieties of *N. tabacum* grown in different agro climatic regions were obtained from Central Tobacco Research Institute (CTRI), Rajahmundry, India. This collection represents different commercial FCV tobacco varieties defined by their morphological and biochemical characteristics (Table 1). 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 [15]. The quantity of the extracted DNA was examined under UV light following agarose gel electrophoresis (0.8% gel containing 0.5 µg/ml ethidium bromide). The DNA concentration was estimated by comparing with known concentration of lambda DNA as standard.

Table 1. Tobacco cultivars investigated in the present study

Sl.	Variety	Parentage	Year of release	Growing state
1	Chatam	Exotic introduction, USA	1950	Andhra Pradesh (AP)
2	Virginia Gold	Exotic introduction,USA	1950	Andhra Pradesh
3	Kanakaprabha	Delcrest x DB 101	1971	AP
4	Dhanadayi	Delcrest x Bisettes	1971	Karnataka and AP
5	FCV Special	Yellow Special x Chatam	1972	Karnataka
6	16/103	Delcrest x Virginia Gold	1972	AP
7	CTRI Special	Local selection	1976	AP
8	Jayasri	Genetic mutant x Structural mutant	1979	AP
9	CTRI Special (MR)	TMVRR 1 x CTRI Special	1980	AP
10	Godavari Special	TMVRR 1 x SP Cross	1981	AP

11	Swarna	Kanakaprabha x Kuofan	1986	Karnataka
12	McNair -12	Exotic introduction, USA	1986	AP
13	Jayasri (MR)	Jayasri x TMVRR 3	1986	AP
14	Hema	Local selection	1987	AP
15	Bhavya	(Virginia 145 x FCV Spl.) x Mutant of FCV Special	1988	Karnataka
16	Gauthami	L-617 x Delcrest	1992	AP
17	CM-12	Chemical mutant of McNair- 12	1993	AP
18	VT 1158	L-617 x CTRI Special (MR)	1993	AP
19	Kanchan	Pure line selection from exotic introduction, USA	1998	Karnataka and AP
20	Thrupthi	L-621 x L-738	1998	Karnataka
21	Rathna	Chemical mutant of FCV Special	2001	Karnataka
22	Hemadri	754-2 # 1 x 16/103	2006	AP
23	Kanthi	Jayasri x L-617	2006	AP
24	Siri	CM-16 x Gauthami	2006	AP

Polymerase chain reaction (PCR)

The SSR markers and their sequences were given in the Table 2. The polymerage chain reaction was carried out in a PTC 100 Peltier thermal cycler, MJ Research, USA. Amplifications were carried out in a 25 µl reaction mixture containing 15 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 10 ng of each of forward and reverse primers, 2.5 µl of 10X buffer and 2.5 mM $MgCl_2$. PCR cycles consisted of one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Final primer extension for 7 min was carried out at 72°C. The PCR products were electrophoresed on 10% polyacrylamide gels [16] to achieve better resolution of the bands. The gels were stained in ethidium bromide for 20 min and documented in Gene Genius bio-imaging system, Syngene, UK. The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Table 2. Data on SSR primer amplification and their sequences

Primer code	Linkage group	Total no. of bands	No. of polymor-phic bands	Polym-orphism(%)	PIC	MI	RP	Hep	Specific bands
PT30111	18	5	3	60	0.435	1.30	1.99	0.286	-
PT30124	4	4	3	75	0.483	1.44	2.49	0.242	-
PT30132	10	6	4	66	0.439	1.75	2.83	0.284	-
PT30150	20	4	1	25	0.5	0.5	1	0.426	-

PT30157	6	5	2	40	0.482	0.964	1.66	0.354	761and 682 bp bands to FCV Special
PT30159	14b	5	2	40	0.409	0.82	1.16	0.428	-
PT30177	22	5	3	60	0.468	1.4	2.41	0.298	-
PT30186	23	2	0	0	0	0	0	0	-
PT30202	7	3	0	0	0	0	0	0	-
PT30205	3b	4	2	50	0.482	0.98	1.66	0.346	190 bp to variety Kanakaprabha
PT30214	14a	3	0	0	0	0	0	0	-
PT30235	21	2	0	0	0	0	0	0	
PT30242	2	4	1	75	0.486	0.49	0.834	0.248	
PT30248	19	4	2	50	0.352	0.71	0.916	0.342	
PT30307	1	3	1	33	0.496	0.49	0.916	0.412	
PT30339	17	4	1	25	0.375	0.38	0.375	0.408	
PT30342	13	4	3	75	0.388	1.17	1.66	0.266	-
PT30350	11	4	3	75	0.369	1.12	1.75	0.242	
PT30380	10	4	2	50	0.274	0.54	0.66	0.325	
PT30416	9	5	2	40	0.361	0.72	0.998	0.348	

PIC, Polymorphic information content; He, Expected heterozygosity; MI, Marker index; RP, Resolving power

Data analysis

All the genotypes were scored for presence or absence of the SSR bands. Only intense 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 [17]. 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. Reliability of clusters on the tree was estimated by bootstrap analysis with 1000 replications to show the degree of confidence of each branch. Only bootstrap values over 50% were considered significant. To analyze the suitability of SSR markers in evaluation of the genetic profile of tobacco, the performance of the marker was measured using polymorphic information content [18], marker index [19], resolving power [20] and expected heterozygosity [21].

Results and discussion

Level of polymorphism

Twenty six tobacco specific microsatellite markers (Simple Sequence repeats) were used in the present study, out of which 20 were chosen for analysis based on clear and well resolved SSR pattern. The number of fragments amplified by each primer ranged from 2 to 6 with an average of 3.9 fragments. A representative SSR profile obtained with PT30157 was given in Fig 1. Maximum number of bands (6) produced by primer PT30132 whereas only two bands were produced by the primers PT30186 and PT30235. Six primers revealed maximum polymorphic bands (above 60%) whereas the primers PT30186, PT30202, PT30214 and PT30235 produced monomorphic pattern. A total of 78 amplicons were produced of which 35 (48%) were polymorphic among 24 varieties. To determine the polymorphic information content (PIC) for each primer, the mean PIC values were analyzed for all loci. High PIC value of 0.5 (PT30150) and low PIC value for PT30380 (0.274) with an average of PIC for primer 0.424 were obtained. They were four primers without PIC values. To determine the general usefulness of the markers used, the marker index (MI) for each SSR primer was calculated. The highest MI was observed with the primer PT30132 and the lowest with the primer PT30339 (0.38), with an average MI of 0.923 per primer was obtained. The resolving power (RP) is a parameter that indicates the discriminatory potential of the primer chosen. The highest RP value was obtained with the primer PT30124 (2.49) and the lowest with the primer PT30339 (0.38). The informativeness of each SSR locus was measured through expected heterogygosity. The expected heterozygosity ranged from 0.242 to 0.428 with an average of 0.328. The SSR markers with high heterozygosity value could be effectively used in FCV tobacco genetic diversity. Siva Raju et al. [11] reported 59.4% of polymorphism among different types of tobacco (42 genotypes) belongs to *N. tabacum* by using RAPD whereas Del Piano *et al*. [10] reported complete monomorphic pattern in 12 *N. tabacum* lines belonging to different types of tobacco. Ren and Timko [4] reported 25.83 % of polymorphism in different types of tobacco by using five primer combinations of AFLP. Yang *et al*. [22] reported 25.43% to 38.56% polymorphic loci in different tobacco types collected from different countries based on inter simple sequence repeats (ISSR) and inter retrotransposon amplification polymorphism (IRAP) markers. High level of SSR polymorphism observed among FCV tobacco varieties of *N. tabacum* in the present study than the levels reported earlier in the same cultivated species could be due to inclusion of local selections, exotic introductions and improved varieties developed by using a wider range of introductions and local lines over a period of three decades.

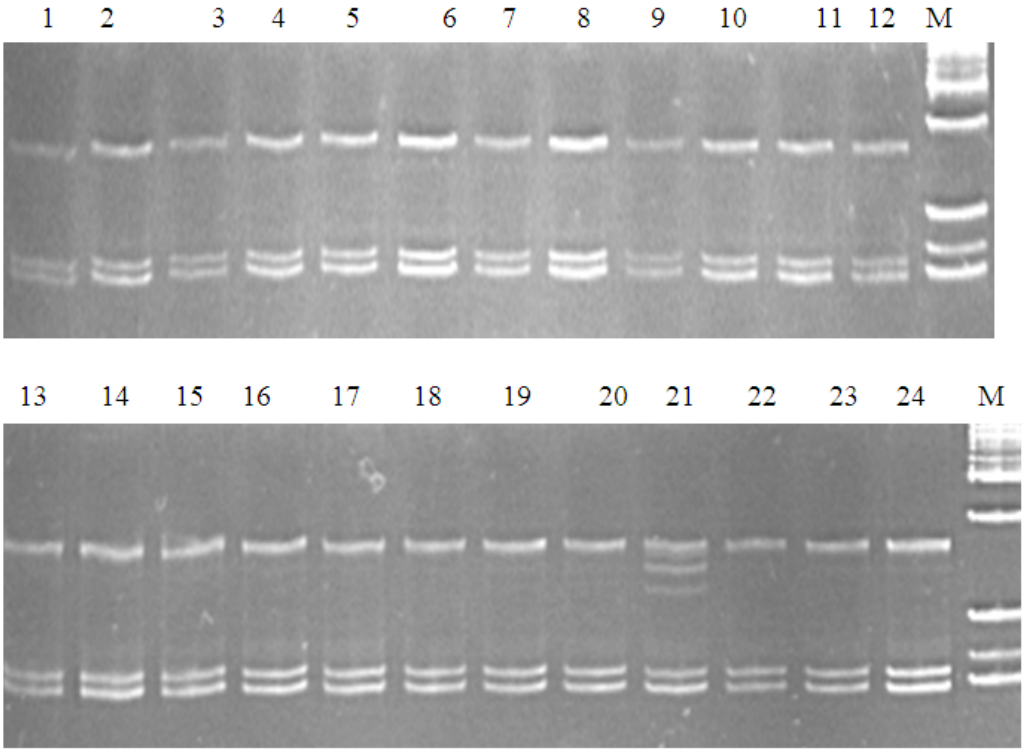

Fig. 1. SSR amplification by PT30157 Lane 1. Virginia Gold, 2. Dhanadayi, 3. Kanakaprabha, 4. CTRI Spl, 5. CTRI Spl MR, 6. Jayasri, 7. Jayasri MR, 8. Godavari Spl, 9. Hema, 10. Gouthami, 11. VT1158, 12.16/103, 13. McNair-12, 14. CM-12, 15. Kanchan, 16. Kanthi, 17. Swarna, 18. Bhavya, 19. Rathna, 20. Thrupthi, 21. FCV Spl, 22. Hemadri, 23. Siri, 24. Chatam and M- DNA ladder

Variety specific markers

None of the primers individually could differentiate all the tobacco varieties used in this study. Some of the primers amplified variety specific fragments that unequally identified the carriers. The primer PT30157 amplified two fragments with 682 and 761 bp specific to the variety FCV special whereas the primer PT30205 produced a fragment with 190 bp specific to the variety Kanakaprabha. The combined profiles based on the polymorphic primers, however provide variety specific patterns and thus could be distinguished some of the varieties.

Genetic diversity among the varieties

The pairwise similarity measures among the varieties ranged from 0.54 to 0.91 revealed a broad genetic base, which could be due to use of divergent parents including exotic introductions and local selections in breeding for different quality and physiological traits. The range of genetic similarity observed among the Indian FCV tobacco varieties was comparatively broader than that reported in a world collection of different tobacco types of this species based on ISSR and IRAP markers [22](Yang *et al*. 2007). The SSR based analysis in the present study thus suggested

that the Indian tobacco breeding has been successful in developing varieties with a relative broader genetic base.

Cluster analysis was performed with Jaccads' similarity matrix and the 24 varieties were separated into two main clusters A and B (Fig. 2). The cluster A was formed by three sub-clusters. In the sub-cluster 1a, the varieties CTRI Special, CTRI Special MR, VT-1158 and Thrupthi were grouped together, where CTRI Special was one of the parents to CTRI Special MR and VT-1158. The varieties Dhanadhayi, Kanakaprabha, Swarna and Kanthi formed the second sub-cluster 2a. The variety Dhanadhayi and Kanakaprabha had line Delcrest as one of the parents. Also, the variety Kanakaprabha was one of the parents to the variety Swarna. The varieties *viz.*, Jayasri and its tobacco mosaic resistant derivative Jayasri MR, variety Hema (a local selection) and variety Godavari Special with different parentages were grouped in third sub-cluster 3a with high bootstrap values. The position of the variety Kanthi above the variety Jayasri was justified as the variety Jayasri was one of the parents to Kanthi. The variety Siri and its parent Gauthami were positioned side by side in the dendrogram.

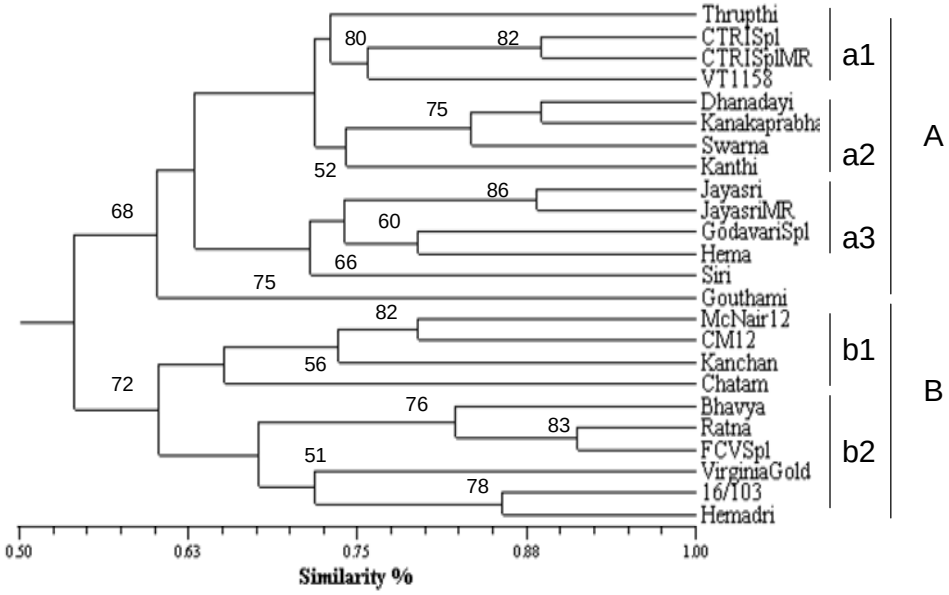

Figure 2. Dendrogram constructed using an UPGMA and SAHN algorithm from Jaccard's similarity data from all the marker profiles revealing genetic relationship among the varieties used in the study.

Cluster B was formed by 10 varieties with two sub-clusters. The varieties McNair12 and its chemical mutant CM 12, Kanchan and Chatam were grouped together in the sub-cluster 1b. The variety FCV Special and its derivatives Bhavya and Ratna (chemical mutant) were grouped and the variety Virginia Gold and its derivative 16/103 and the variety Hemadri, derivative of 16/103 were grouped together with 85% of genetic similarity in sub-cluster 2b with high bootstrap values. Thus the grouping of the varieties in the sub-clusters was mainly based on parentage. The dendrogram revealed that the genotypes that are derivatives of genetically similar

varieties clustered more together. In the cluster A, the varieties developed indigenously by different breeding methods were clustered whereas in the cluster B, the exotic introductions and their derivatives were grouped together.

The mean genetic similarity among the indigenously developed cultivars was 72%, suggesting that the use of local selection for breeding has not been effective in significant broadening of the genetic base of commercial FCV tobacco types. However the diversity level observed in this study is higher than that of *N. tabacum* types studied earlier [4,10,11]. This narrow genetic variation may also be attributed to the preferential use of some varieties as parents in the breeding program because of prepotency of these varieties to produce desirable qualitative traits besides their adaptability to the different agro climatic conditions.

The High level of genetic similarity (78.5%) among the varieties Jayasri MR, CTRI Special, CTRI special MR, Godavari Special and VT 1158 may be due to ancestral background of variety FCV Special and the grouping was also supported by bootstrap values. Another set of varieties *viz*. Gauthami, Kanakaprabha, Dhanadayi and Jayasri MR have developed in the background of variety Delcrest. The close genetic similarity (>80%) among the varieties Dhanadayi, Kanakaprabha, CTRI Special, CTRI Special MR, Jayasri, Jayasri MR was due to use of one or other among them as parents or have a common parent. The variety Dhanadayi showed very low genetic diversity (<50%) with the varieties Bhavya, Ratna, FCV Special and chatam as it had different parentage. For incorporation TMV resistance, the line TMVRR1 was used in the development of variety Godavari special, TMVRR2 used for development of CTRI special MR and TMVV44-3 (derivative of TMVRR1) was used in the development of Jayasri MR. Thus these FCV varieties developed are genetically and were clustered together in the analysis. The analysis of genetic variation both within and among elite breeding material and varieties is of fundamental interest in breeding. This helps planning appropriate breeding programs and to predict potential genetic gain.

Extensive amount of morphological and cytological information were earlier used in estimating evolutionary relationship in the genus *Nicotiana* [23]. However, little information is available on the extent of genetic variation among commercially cultivated Indian FCV tobacco of *N. tabacum*. In this study, we used SSR analysis to gain insight into the degree of intraspecific variations in the FCV cultivars of *N. tabacum*. The results indicated that with specific selective primers, it is possible to yield a specific number of polymorphic fragments to allow meaningful comparison among cultivated FCV tobacco genotypes.

In conclusion, the large range of similarity values for related cultivars using microsatellites provides greater confidence for assessment of genetic diversity and relationships. The level of genetic diversity within the cultivated FCV tobacco varieties was higher than that reported [11]. Genetic relationship among the FCV tobacco varieties based on polymorphic DNA profile largely corresponded with parentage. The practical approach developed in the study is useful in DNA fingerprinting also. The variety -specific markers identified in this study would be useful in testing genetic purity of seeds of commercial FCV tobacco varieties.

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