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Molecular Diversity in Indian Tobacco Types as Revealed by Randomly Amplified DNA Polymorphism

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During the past five decades, a large number of tobacco varieties have been developed for different end uses in India through pure line selection from local land races, mutation breeding, and hybridization involving local selections and exotic introductions followed by pedigree selection. No systematic effort has been made to understand the existing diversity pattern in these varieties, which is crucial to define future breeding strategy in this important commercial crop. We characterized 46 varieties belonging to 10 different manufacturing tobacco types cultivated under different agro-climatic conditions in India along with two wild species of *Nicotiana* using 40 arbitrary primers in RAPD. The level of polymorphism among the varieties of *N. tabacum* was 59.4%, which was more than double the level observed in the other cultivated species *N. rustica* (25.2%). A broader range (0.64 to 0.94) of pair wise similarity measures in *N. tabacum* than in *N. rustica* (0.83 to 0.92) reflected the more diversified breeding efforts in the major cultivated species. The two wild species namely, *N. glutinosa* and *N. gossei* clustered separately from the two cultivated species. Molecular classification of the varieties corresponded largely with their manufacturing trait and parentage. RAPD markers provided sufficient resolution to distinguish among closely related tobacco types. Nine RAPD markers were found conserved across all the varieties and species. The markers found specific to the varieties can be used in correct identification of the carrier genotypes in trade and commerce. This is the first report on the molecular diversity analysis of Indian tobacco.

***Key words**: Nicotiana* spp, tobacco, RAPD markers, genetic diversity, variety specific markers.

The genus *Nicotiana* is a member of the family *Solanaceae*. Out of 64 recognized species (1), two species namely, *N. tabacum* and *N. 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. These types are defined to a large extent by the method of curing, intended use in manufacturing, and by some distinct morphological and chemical characteristics. The types of tobacco grown at different locations differ considerably depending on the climatic conditions and nutrient supply. Hence breeding and selection of tobacco varieties is specific to the intended use and location, right from the choice of parents to the final stages of evaluation and selection.

Prior to 1970, tobacco cultivation in India was confined to a few introductions such as Harrison special, Chatam, Delcrest and Virginia Gold. During 1970s the emphasis was on pure line selection from the local land races, which led to the development of popular varieties such as CTRI Special (FCV), Hema (FCV) and DP-401 (chewing). During this period, cross hybridization involving the introductions and local selections was also carried out leading to release of many high yielding varieties including Kanakaprabha, Gauthami and VT 1158 (all FCV). After 1980, emphasis shifted towards development of varieties for biotic and abiotic stress tolerance through recombination breeding and induced mutations. This approach resulted in the development of many stress tolerant varieties including Jayasree MR (tobacco mosaic virus resistant), Bhavya (Black shank and root knot nematode resistant) and Lanka Special (Powdery mildew resistant). Varieties suited for different end users and locations such as Viswanath (Natu), Sendarpatty Special (cheroot), GT-6 (Bidi) and Meenakshi (chewing) were also developed (2). Most of the Indian tobacco varieties of FCV and burley type contain lower levels of carcinogenic substances like nicotine (1-2%) and tobacco specific nitrosoamines (TSNA) (0.42 to 1.44ppm)

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as compared to tobacco from other countries (3). Though a large number of varieties belonging to different commercial types have been developed in India no information is available on their genetic base.

Molecular genetic markers have become useful tools to provide a relatively unbiased estimation of genetic diversity in plants (4). PCR based markers like RAPD are being used in the analysis of genetic diversity in crop plants because of the relative ease with which PCR assays can be carried out compared to the other markers. Besides, prior knowledge about the genome is also not a pre-requisite, which makes RAPD a common method for such studies in different crops. In tobacco, RAPD has been used mainly to identify markers linked to genes for resistance to pathogens (5-7). Del Piano *et al* (8) carried out a preliminary analysis of genetic diversity in 12 varieties of *N. tabacum* using 3 random primers in RAPD. Till date, no systematic effort has been made to understand the existing diversity pattern in the Indian tobacco varieties, which is crucial to define future breeding strategy in this important commercial crop. This is the first effort to study genetic diversity using molecular markers among 46 tobacco varieties belonging to *N. tabacum* and *N. rustica,* currently being cultivated in different agro-climatic zones of India and two wild species, which have been used as donors of biotic stress tolerance genes.

Materials and Methods

Plant material — Seeds of 41 varieties of *N. tabacum* and five varieties of *N. rustica* grown in different agro climatic regions were obtained from Central Tobacco Research Institute (CTRI), Rajahmundry, India. This collection represents different commercial types of tobacco defined by their intended manufacturing (cigar filler, cigar wrapper and bidi), method of curing (air-cured and flue-cured) and morphological and biochemical characteristics (natu and chewing). The wild species *N. glutinosa* and *N. gossei,* which are being used as donors of biotic stress tolerance genes, were included for comparison (Table 1).

DNA extraction and RAPD assay — Thirty-day-old seedlings of each variety were collected, bulked, frozen in liquid nitrogen, and stored at -80°C until used for DNA extraction. DNA was extracted from pulverized frozen seedlings as per Doyle and Doyle (9) and quantified on 0.8% agarose gel. The PCR amplification conditions were as described by Williams *et al* (10) with minor modifications. Amplifications were carried out in a 25 µl reaction mixture containing 15 ng template DNA, 0.5 units of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.2 mM of each dNTP and 6 picomoles of each primer. Sixty random primers (Operon Technologies, Alameda, CA, USA) were used for amplification. Thermal profile consisted of 45 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min, followed by extension for 7 min at 72°C in a thermal cycler (Biometra, Gottingen, Germany). Fourteen µl of the amplification product was electrophoresed on 1.2% agarose gel in 1x TBE buffer and stained using ethidium bromide. The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

Data analysis — Clearly resolved bands were scored for presence (1) or absence (0). The data on 439 bands generated by 40 primers were selected for the analysis of genetic diversity. The NTSYS-pc software version 2.02 (11) was used to calculate Jaccard's (12) similarity coefficients. Based on UPGMA and SAHN clustering, a dendrogram depicting the genetic relationship among the varieties was prepared. Mean similarity of individual varieties with the rest and among the varieties within a particular cluster was computed from the similarity matrix table. Principal coordinate analysis (PCO) was also carried out to show the distribution of the varieties in scatter-plot (13). Bootstrap analysis (500 iterations) of the binary data was done using the WINBOOT programme (14) to determine the confidence limits of the UPGMA clusters.

Results and Discussion

Level of polymorphism — Sixty random decamer primers were used in this study, out of which 40 were chosen for analysis based on clear and well-resolved RAPD pattern. The number of fragments amplified by each primer ranged from 2 to 16 with an average of 6.9 and 4.1 fragments in *N. tabacum* and *N. rustica,* respectively. A representative RAPD profile obtained with the primer OPB13 is given in Fig. 1. Maximum number of bands (16 and 12 respectively) was produced in the two cultivated species by the primer OPAA5. The least number (2) of bands were produced by the primer OPAC15 in *N. tabacum* and OPAC13 in *N. rustica*. The number of fragments amplified in *N. tabacum* is comparable to that reported earlier (8).

All the forty primers were polymorphic across the four species. In the two cultivated species, four primers namely

Table 1. Tobacco varieties used in RAPD analysis

Sl. No.	Species	Variety	Parentage	Type	Use	Year of release	Area of cultivation
1	*N. tabacum*	CTRI special	Local selection	FCV	Cigarette	1976	Andhra Pradesh
2		CTRI special MR	TMVRR-2 X CTRI spl.	FCV	Cigarette	1980	Andhra Pradesh
3		Jayasri	(Del. X Hicks) X CTRI spl.	FCV	Cigarette	1979	Andhra Pradesh
4		Jayasri MR	TMV44-3 X Jayasri	FCV	Cigarette	1986	Andhra Pradesh
5		VT 1158	L 617 X CTRI spl.	FCV	Cigarette	1993	Andhra Pradesh
6		HEMA	Local Selection	FCV	Cigarette	1987	Andhra Pradesh
7		Cy79	Jayasri X L617	FCV	Cigarette	2001	Andhra Pradesh
8		CM 12	Chemical Mutant of Mc Nair 12	FCV	Cigarette	1993	Andhra Pradesh
9		MC Nair 12	Exotic Introduction	FCV	Cigarette	1986	Andhra Pradesh
10		NLS-4	Exotic Introduction	FCV	Cigarette	1998	Andhra Pradesh
11		Gouthami	L 617x Delcrest	FCV	Cigarette	1982	Andhra Pradesh
12		Bhavya	FCV Sp. X Virgina 145	FCV	Cigarette	1987	Karnataka
13		Ratna	FCV Spl. Mutant	FCV	Cigarette	2001	Karnataka
14		FCV special	V. Spl. X Chatham	FCV	Cigarette	1976	Karnataka
15		KST- 19	L 621 X L 738	FCV	Cigarette	1998	Karnataka
16		Lanka special	DR1 X Kuofon	Lanka	Cigarette	1981	Andhra Pradesh
17		Burley 21	Exotic Introduction	Burley	Cigarette	1980	Andhra Pradesh
18		BSR B2	Delcrest(4X)N.PlumbaginifoliaX La Burley 21x Burley 58	Burley	Cigarette	2001	Andhra Pradesh
19		Natu special	KVT X Toka AKU	Natu	Cigarette	1982	Andhra Pradesh
20		Viswanath	I 452 X Karuvazhai	Natu	Cigarette	1986	Andhra Pradesh
21		Pyruvithanam	Local Selection	Natu	Cigarette	2002	Andhra Pradesh
22		Jatichama	Jati local type	Hookah	Hookah	1956	West Bangal
23		Dixie shade	Exotic Introduction	Cigar Wrapper	Cigar Wrapper	1974	West Bangal
24		S-5	Dixie shade X Rangpur Sumatra	Cigar Wrapper	Cigar Wrapper	1977	West Bangal
25		Krishna	VV-2 X Maryland	Cigar filler	Cigar filler	1985	Tamil Nadu
26		Sendarapatty special	OK-1 X VV2	Cheroot	Cheroot	1986	Tamil Nadu
27		Bhagya Sri	PL-16 X L 738	Bidi	Bidi	1994	Karnataka
28		GT 6	783-51-11-13-61 X Smyrna	Bidi	Bidi	1984	Gujarat
29		GT 7	930-47 X Anand 2	Bidi	Bidi	1993	Gujarat
30		NPN – 190	(169-119 X Olor) X 121-1	Bidi	Bidi	1979	Gujarat
31		Anand 2	Sokhadu X 88-47	Bidi	Bidi	1984	Gujarat
32		Viram	I 64 X VTK – I	Chewing	Chewing	1977	Tamil Nadu
33		Bhagyalakshmi	I 64 X I- 735	Chewing	Chewing	1980	Tamil Nadu
34		Meenakshi	PV-7 X HV67-9	Chewing	Chewing	1982	Tamil Nadu
35		Abhirami	I-64 Mutant	Chewing	Chewing	2001	Tamil Nadu
36		Pusa tobacco (PT-76)	HP 60-1 X Bori	Chewing	Chewing	1990	Bihar
37		Gandak bahar	NP 35 X Bori Rampur	Chewing	Chewing	1976	Bihar
38		Sona	Borimalinagar tohra X DP-401	Chewing	Chewing	1977	Bihar
39		Vaishali special	Bandi X Sona	Chewing	Chewing	1993	Bihar
40		DP 401	Local Variety	Chewing	Chewing	1961	Bihar
41		Lichchavi	Sona X Boritepari	Chewing	Chewing	2001	Bihar
42	*N. rustica*	HD65-40	BVH-5 X Sel 47	Hookah	Hookah	1977	West Bangal
43		DD-437	Selection from Hemti Bulk Crop	Hookah	Hookah	1977	West Bangal
44		Dharala	C304 X DD437	Hookah	Hookah	2001	West Bangal
45		GC 1	Local Selection	Chewing	Chewing	1981	Gujarat
46		GC 2	GC 1 X Coker 1	Chewing	Chewing	1994	Gujarat
47	*N. glutinosa*	Wild accession	-	-	-	-	-
48	*N. gossei*	Wild accession	-	-	-	-	-

FCV - Flue-cured Virginia; - Not Known

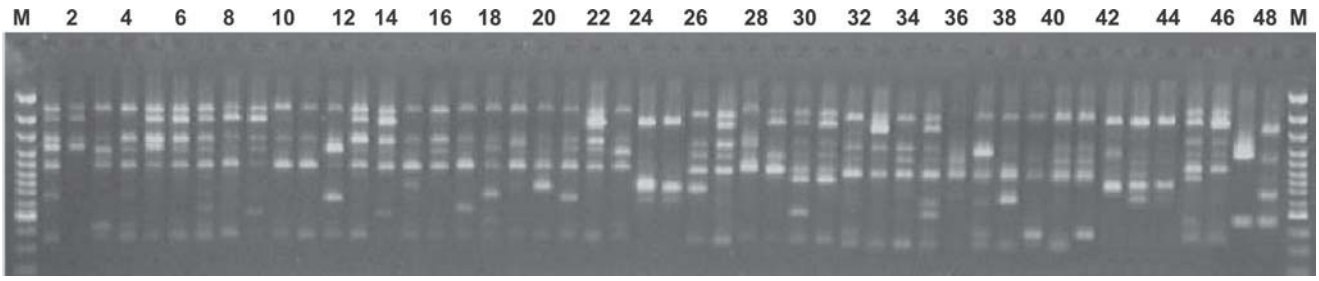

Fig. 1. RAPD profiles of 46 cultivated lines of *N. tabacum* and *N. rustica* and 2 wild species (*N. glutinosa* and *N. gossei*) obtained with the primer OPB13. The lane numbers 1 to 48 correspond to the lines as listed in the Table 1. M - 50 bp DNA ladder.

OPA5, OPAC10, OPAB1 and OPP4 revealed maximum polymorphic fragments (8), where as primers OPA8 and OPD7 revealed minimum polymorphic fragments (2). A total of 439 bands were amplified of which 430 (97.94%) were polymorphic across the four species. The level of polymorphism among the varieties of *N. tabacum* was 59.4%, which was more than double the level observed in the other cultivated species *N. rustica* (25.2%). Del Piano *et al* (8) reported complete monomorphic pattern in 12 *N. tabacum* lines belonging to different types using RAPD. Ren and Timko (15) used five primer combinations in AFLP to amplify 460 fragments out of which 119 (25.83%) were polymorphic in *N. tabacum* varieties. In a recent study, Yang *et al* (16) reported 25.43% to 38.56% polymorphic loci in different tobacco types collected from different countries including USA, China, Canada and Turkey based on inter simple sequence repeat (ISSR) and inter retrotransposon amplification polymorphism (IRAP) markers. The observed level of polymorphism was also higher than that reported in Australian potato cultivars (12.8%) (17) but lower than egg plant (72.4%) (18), which are members of the same family *Solanacea*. Higher level of RAPD polymorphism observed in the Indian varieties of *N. tabacum* in the present study than the levels reported earlier in the same cultivated species could be due to inclusion in our study of local selections, exotic introductions and improved varieties developed by using of a wider range of introductions and local lines over a period of three decades. Breeding efforts in *N. rustica* has not been as diversified as in *N. tabacum*, which was amply reflected in lesser number of varieties developed and significantly lower level of polymorphism detected in this species.

Variety specific markers — None of the primers individually could differentiate all the tobacco varieties used in the study. The combined profiles based on the polymorphic primers, however provided variety specific patterns and thus could distinguish all the varieties from each other. Some of the primers amplified variety specific fragments that uniquely identified the carriers. For instance, primer OPA8, OPAC10, OPC11 and OPJ15 produced bands of 1200, 2200, 925 and 800 bp size in the varieties Hema, DP 401, Dixie shade and Bhavya, respectively. The Primer OPA8 produced a band of 450 bp size specific to all non-FCV types. These variety or group specific patterns/ markers would be useful in correct identification of the genotypes in trade and commerce.

Genetic relationship among the species — The genetic similarity between the two cultivated species of *Nicotiana* was low (21.3%), which is clearly in accordance with the earlier classification of these two species in to different subgenus of *Nicotiana* (19). They possibly evolved from two divergent progenitors and a lack of crossability between them has not allowed gene flow. However, these two species shared 29 bands in common, which have remained conserved in evolution. Sequence analysis of multiple nuclear fragments from such potentially conserved regions of the genome would be useful for understanding phylogenetic relationship and the pattern of divergence in the genus *Nicotiana*. The wild species *N. glutinosa* showed relatively closer (47.7%) similarity to *N. tabacum* than *N. gossei* that had 45.7% similarity with the major cultivated species. In contrast, the other cultivated species *N. rustica* had 58% similarity with the two wild species that suggested closer evolutionary relationship of the two wild species with *N. rustica* than with *N. tabacum* (19).

Genetic diversity among the varieties — The pair wise similarity measures among the varieties ranged from 0.638 to 0.937 in *N. tabacum* and from 0.832 to 0.919 in *N. rustica*. This revealed a broader genetic base of the varieties in *N. tabacum* than in *N. rustica,* which could be due to use of divergent parents including exotic introductions and local selections in breeding for different end uses in *N. tabacum*

as compared to *N. rustica* (see Table 1). The *N. tabacum* varieties remained together in the cluster A (Fig. 2) at an average similarity of 78.3%, while those of *N. rustica* remained in the cluster C with an average similarity of 88%. These major clusters were supported by high bootstrap values (Fig. 2). The result of cluster analysis corresponded well with that from principal coordinate analysis (data not shown) further supporting genetic isolation between the two species. The range of genetic diversity observed among the Indian *N. tabacum* varieties is comparatively broader than that reported in a world collection of different tobacco types of this species (70-90 % similarity) based on ISSR and IRAP markers (16). The RAPD based analysis in the present study thus suggested that the Indian tobacco breeding effort has been successful in developing varieties with a relatively broader genetic base than those developed in other countries. This, however, needs further validation using a more robust marker system than RAPD.

The parentage of varieties contributed to the grouping pattern in both the cultivated species, majority of which were supported by ≥50 bootstrap values (Fig. 2). In case of *N. tabacum* the varieties CTRI Special and CTRI Special MR, which is a derivative of CTRI Special, were grouped together (sub-cluster 1, bootstrap value 100). Similarly, in the sub-cluster 2, the varieties Jayasri and its virus resistant derivative Jayasri MR (bootstrap 33), and Mc Nair 12 and its chemical mutant CM12 (bootstrap 80) were grouped together. In sub-cluster 4, variety FCV Special and its derivatives Ratna (chemical mutant) (bootstrap 57) and Bhavya (bootstrap 64) were together. Though the varieties of chewing tobacco grouped based upon manufacturing trait, they remained divided into sub-clusters due to their parentage. The Variety Dixie shade (cigar wrapper) and one of its parents, S-5 were grouped together (sub-cluster 7, bootstrap 37). Variety Krishna (cigar filler) and Sendarapatty special (cheroot) remained close to each other due to common parentage (bootstrap 50). Though

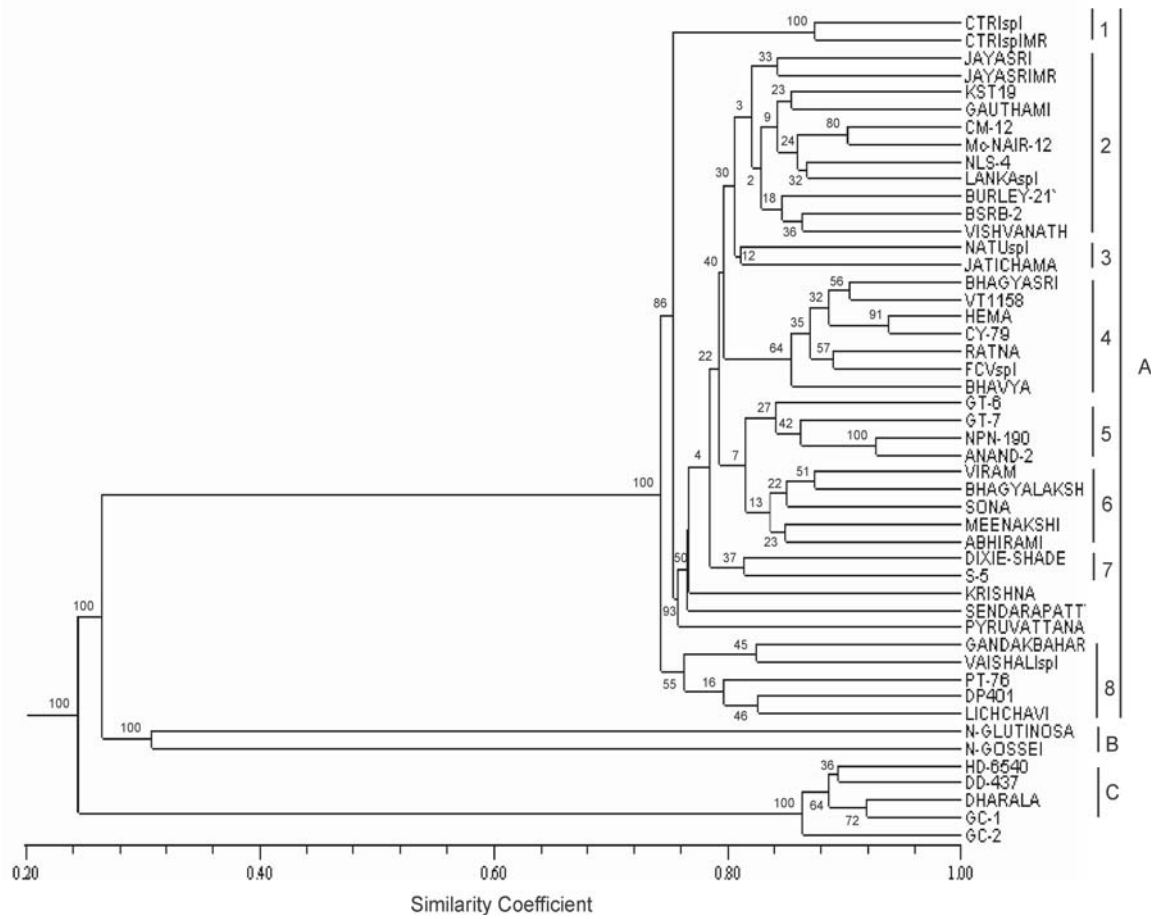

Fig. 2. Dendrogram showing genetic relationship among the 46 varieties belonging to two cultivated tobacco species and two accessions of the wild relatives *N. glutinosa* and *N. gossei* based on 430 polymorphic fragments obtained with 40 random primers in RAPD.

the varieties of *N. rustica* had different end uses such as GC1 and GC2 for chewing and DD437 and Dharala for hookah, they grouped in the same cluster supported mostly by >50 bootstrap values because of their related pedigree. Cao *et al* (20) reported grouping of wheat cultivars with similar pedigrees than those known to be unrelated by using 31 RAPD primers. Similarly, 54 flax cultivars were also grouped based on parentage using RAPD markers (21).

Sub-grouping of the varieties within the main cluster also corresponded with the manufacturing trait with exceptions although supported by lower bootstrap values (Fig. 2). Out of 21 varieties used for cigarette manufacturing, two remained together in the sub-cluster 1, 11 in sub-cluster 2 and 6 in the sub-cluster 4, while the rest two either grouped in another sub-cluster (Natu spl in sub-cluster 3) or remained isolated from others (Pyruvithanam). Four of the five bidi types remained in the sub-cluster 5 and one (Bhagyalaksmi) grouped with cigarette type in sub-cluster 4. Of the 10-chwieng types, 5 each were grouped in sub-clusters 6 and 8. The sub-cluster 7 had two of the cigar wrapper types. One each of cigar filler (Krishna) and cheroot (Sendarpatty spl) remained isolated from others. Grouping of varieties based on manufacturing traits with exceptions has also been reported by Ren and Timko (15) from AFLP studies in tobacco.

In conclusion, our study revealed that the RAPD assay was efficient in differentiation of species as well as varieties within the two cultivated species of *Nicotiana.* The level of genetic diversity within the cultivated species *N. tabaccum* was much higher than that in *N. rustica* that reflected the more diversified breeding efforts in the major cultivated species in India. Genetic relationship among the tobacco varieties based on polymorphic DNA profiles largely corresponded with parentage and manufacturing trait. The variety-specific markers identified in the study would be useful in testing of purity of seeds of commercial tobacco varieties.

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