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Genetic Diversity among Flue-cured Tobacco Cultivars Based on RAPD and AFLP Markers

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ABSTRACT

The aim of this work was to study the genetic diversity among flue-cured tobacco cultivars. RAPD and AFLP analyses were used to assess the genetic similarity among selected accessions of flue-cured tobacco. Seventy eight RAPD and 154 AFLP polymorphic bands were obtained and used to assess the genetic diversity among 28 tobacco accessions. The cultivar relationships were estimated through the cluster analysis (UPGMA) based on RAPD data and AFLP data. The accessions were grouped into three major clusters and these shared common ancestry clustered together.

Key words: Flue-cured tobacco, RAPD, AFLP, genetic diversity, DNA polymorphism

INTRODUCTION

The flue-cured tobacco *(Nicotiana tabacum L.)* is one of the most important types for the tobacco production in the world. Undoubtedly, the study of the genetic diversity of flue-cured tobacco cultivars is important not only for the germplasm conservation but also in parental choice for breeding purposes. The RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) are genetic fingerprinting techniques suitable for the genetic evaluation of flue-cured tobacco. The techniques have been successfully used to genetically analyze many different plant species (Crochemore et al., 2003; Diniz et al., 2005; Ni et al., 2006 and Yang et al., 2006).

In this work, the RAPD and AFLP procedures were used to assess the amount of polymorphisms detected among the flue-cured tobacco cultivars and to estimate the relationships.

MATERIALS AND METHODS

Plant Materials

The seeds of the flue-cured tobacco were obtained from the germplasm collection of the South Center Tobacco Breeding Research of China in Yunnan province, southwest of China. The collection was consisted of 298 cultivars and breeding lines from different places. On the basis of results from the field trials conducted at Yuxi, Yunnan, from 1994 to 1996 (Lei et al., 1997), 28 accessions with desirable agronomic characteristics, such as large leaf size, high leaf yields, low nicotine contains, or resistance to various diseases or insects, were selected for evaluation in this study. These

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accessions represented the genotypes likely to be used in future flue-cured tobacco breeding efforts in south China. The name and origin of the cultivar were showed in the Table 1.

The seeds were planted in the pots and grown in the greenhouse at 28 to 32 °C. Twenty days after the germination, the shoots were harvested from 40 seedlings of each accession. The DNA was extracted from the shoots by the CTAB method (De Riek et al., 2001).

RAPD analysis

The amplification was performed in 20μL solution containing 2μL of the 10×buffer, and 100 mM each of dNTPs, 0.4 mM primer, 25 ng genomic DNA, and 1 unit of Taq polymerase. The reaction mixture was overlaid with 40μL mineral oil. The amplifications were carried out using a 2400 Perkin-Elmer (Perkin Elmer, USA) thermal cycler programmed for 40 cycles as follows: 30 s at 94°C, 30 s at 36 °C, 1.5 min at 72 °C, with an initial melting of 6 min at 94 °C, and a final extension of 6 min at 72 °C. The amplification products were analyzed by the electrophoresis in a 1.5 % agarose gel with 1×TAE buffer (0.004 M Tris-acetate and 0.002 M EDTA).

Table 1- Flue-cured tobacco cultivars with pedigree and origin used in RAPD and AFLP analysis.

Number	Cultivar	Pedigree	Origin
1	Changbohuang	Unknown	Henan(CHN)
2	NC82	6129×Coker319	USA
3	581	Chujingyan	Yunnan(CHN)
4	Yunyan 3	Zhaojie 8 dui×Coker347	Yunnan(CHN)
5	Jingtai33	Unknown	Shangxi(CHN)
6	Yunyan 86	Yunyan 2 ×K326	Yunnan(CHN)
7	Yunyan 76	Speight G-28×K326	Yunnan(CHN)
8	Jingyehuang	Changbohuang	Henan(CHN)
9	82-3041	Speight G-28×Burley599	Henan(CHN)
10	Zhongyan 86	Speight G-28×Jingyehuang	Henan(CHN)
11	Jiyan 5	Jingyehuang×Coker86	Jiling(CHN)
12	CV87	（CV58×Speight G-28）×[CV58×（Speight G-28×NC82）F1]	Henan(CHN)
13	77809-12	（Lingi 1 ×Virginia 115）F6	Yunnan(CHN)
14	Qingsheng 2	Jingyehuang	Henan(CHN)
15	Yunyan 84	Yunyan 2×K326	Yunnan(CHN)
16	Chunjingyan	Gold Dollar	Yunnan(CHN)
17	Jingxing 6007	Jingxing	Henan(CHN)
18	RG11	NC50×K399	USA
19	Zhongyan 14	Jingxing6007×Speight G-28	Henan(CHN)
20	Yunyan 1	Gold Dollar	Yunnan(CHN)
21	K394	Speight G-28×McNair944	USA
22	Xiaohuangjing1025	Xiaohuangjing	Shangdong(CHN)
23	8813	Unknown	Shichuan(CHN)
24	Yunyan 2	Red Flowers Gold Dollar×Speight G-28	Yunnan(CHN)
25	CV85	（CV58×Speight G-28）×[CV58×（Speight G-28×NC82）F1]	Henan(CHN)
26	CV73	（CV58×Speight G-28）×[CV58×（Speight G-28×NC82）F1]	Henan(CHN)
27	Oxford 26	TI 448A×400	USA
28	K149	[Speight G-28×Coker254] ×（CB139×F-105）×（Speight G-28×Coker254）] ×McN399	USA

AFLP analysis

The AFLP analysis was performed following the manufacturer's protocol (Life Technologies). The DNA was digested simultaneously with restriction enzymes *Eco*RI and *Mse*I. The selective amplifications were performed using the primer pairs listed in Table 2. The restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters. The primers within set *Eco*RI included the sequence 5'- GAC TGC GTA CCA ATT C; primers of the *Mse*I set had the sequence 5'-GAT GAG TCC TGA GTA A. The pre and the selective amplifications were performed in a 2400 Perkin-Elmer Thermocycler. An equal volume (2μL) of loading dye (95% v/v formamide and 0.08% w/v bromophenol blue, 20 mM EDTA) was added to each sample, which was then denatured at 95°C for 3 min and placed on the ice for 2 min before loading. The amplification products were analyzed by the electrophoresis in a 6.5% polyacrylamide

gel. The electrophoresis parameters were set to 1500 V, 40.0 mA, 40.0 W, 50°C and the run time was set to 2.0 h. Separated AFLP products were visualized using silver staining as described in the Promega Silver Staining kit and gel images were saved as TIF files for analysis.

Data analysis

Each accession was scored 1 for the presence or 0 for the absence of each polymorphic band. The bands present in all accessions were not scored.

Only bright, clearly distinguishable bands were used in the genetic analysis. All the statistical analyses were performed by NTSYS-pc, Version 1.8. Similarity matrices (data not shown) were constructed from the binary data with Jaccard's coefficients (Jaccard, 1908). The dendrograms were generated with the unweighted pair-group method, arithmetic average (UPGMA) algorithm as described by Sneath and Sokal (1973).

RESULTS AND DISCUSSION

From the 200 primers used in RAPD analysis, 63 (31.5%) produced the amplification products that were too faint to score or could not be consistently reproduced, and 124 (62%) produced monomorphic banding patterns. Only 13 (6.5%) out of 200 primers were scored. A total of 125 bands were scored from the comparison of amplifications with 13 primers of DNAs from 28 flue-cured tobacco cultivars, with an average of 9.6 bands scored per primer (Table 2.). The polymorphic bands were 78 (62.4%), and one primer detected a mean of 6 polymorphic bands per reaction.

Fourteen selective AFLP primers were screened against all 28 accessions. Four primer pairs were not included in the final analysis because either the amplification profile was consistently too faint to score accurately (AAC/CGC) or only monomorphic amplification products were produced (AAC/CTG, ACT/CTC, ACT/CTG). The ten informative primer pairs used in the final analysis were listed in Table 2. A total of 154 fragments were analyzed using the ten pairs of primers. Five hundred and sixty-one fragments were scored in the assay performed by using the ten pairs, with an average of 56.1 fragments per pair of primers used. One hundred and fifty-four fragments were polymorphic, with an average of 15.4 per reaction, with 27.45% polymorphism.

Table 2- DNA fragment polymorphism in flue-cured cultivars after RAPD and AFLP analysis.

RAPD Primer	Total bands	Polymorphic bands	Polymorphism %	AFLP Primer pair	Total bands	Polymorphic bands	Polymorphism %
OPA 03	3	2		ACC/CAG	52	12	
OPA 04	10	4		ACC/CTG	76	26	
OPA 07	13	9		ACC/CAT	66	25	
OPB 01	10	6		ACC/CAA	39	15	
OPB 13	6	4		ACC/CTT	49	3	
OPC 01	7	4		AAC/CAT	64	11	
OPD 05	8	5		AAC/CAG	38	1	
OPD 14	8	5		AAG/CAA	47	3	
OPK 01	6	5		AAG/CAG	81	26	
OPL 03	14	9		AAG/CTT	53	32	
OPL 09	14	8					
OPW 05	11	10					
OPY 10	15	7					
Total	125	78	62.4%		561	154	27.45%
Average	9.6	6			56.1	15.4	

Results from cluster analyses using RAPD or AFLP data indicated that these two marker techniques provided similar, but not identical information (data not shown). For example, Zhongyan 86 was the offspring of both Speight G-28 and Jingyehuang, in the dendrogram based on RAPD data, it was clustered with the progenies of Jingyehuang; it was grouped with the progenies of Speight G-28, in the dendrogram based on AFLP data. This observation could be related to the larger number of AFLP bands used in the analyses compared with the number of RAPD bands used.

The RAPD and AFLP data were combined to generate a dendrogram incorporating both types of the DNA marker data, the relationships among the accessions analyzed are shown in Fig. 1. The RAPD and AFLP based Jaccard's similarity coefficients ranged from 0.13 to 0.88. The

accessions were grouped into three major clusters. Group I included 17.86% of the accessions; group II included 64.29% of the accessions; group III included 17.86% of the accessions.

The dendrogram did not indicate any clear pattern of division among the flue-cured tobacco accessions based on the geographic origin, as seen in some other crops (Paul et al., 1997; Spooner et al., 1996). However, those accessions that shared common ancestry clustered together. For example, the cultivars bred by Speight G-28 such as 82-3041, K149, K394, Zhongyan 14, CV73, CV85 and CV87 (Table 1) were clustered together in Group II; Jiyan 5 and Qingsheng 2 were the offsprings of the Jingyehuang, Jingyehuang was bred by Changbohuang (Table 1), they were all clustered with Jingyehuang in Group II; Yunyan 2, chunjingyan and Yunyan1 were bred by Gold Dollar (Table 1), they were clustered together in Group I; and one of the crossing parents of Yunyan 76, Yunyan 84 and Yunyan 86 was K326 (Table 1), they were also clustered together in GroupII.

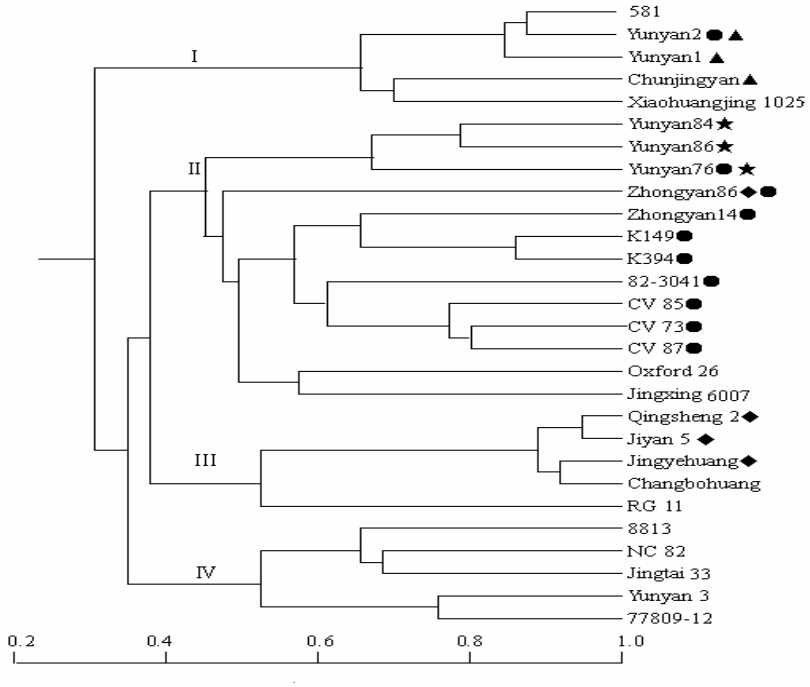

Figure 1 - Dendrogram of the 24 flue-cured tobacco cultivars from cluster analysis (UPGMA) of the genetic distances, on the basis of RAPD and AFLP data. Symbols indicate ancestry of origin: ♦= Jingyehuang. ●= Speight G-28. ▲= Gold Dollar. ★=K326.

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