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Genetic diversity among flue-cured tobacco (*Nicotiana tabacum* L.) revealed by amplified fragment length polymorphism

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ABSTRACT. Flue-cured tobacco (*Nicotiana tabacum* L.) is one of the most important commercial crops in the world. Genetic diversity studies provide estimates on the level of genetic variation among diverse materials that can be used in germplasm management, varietal protection, and flue-cured tobacco improvement. Amplified fragment length polymorphism (AFLP) analysis of 51 flue-cured tobacco cultivars produced a total of 1479 unambiguous DNA fragments. Cluster analyses using the unweighted pair group method with arithmetic mean (UPGMA) showed that the cultivars could be grouped into American or Chinese types, with the Chinese types being further clustered into four subgroups and American ones into two subgroups. The average pairwise genetic distance was 0.167 and ranged from 0.024 to 0.267. AMOVA analysis showed that 55.76% of genetic variation came from cultivars having different origins and 44.24% from cultivars having the same origin. The overall average Fst of the 51 accessions was 0.177. Mean Fst of each accession against the rest ranged from 0.152 to 0.238. The findings of this study revealed that the present day commonly grown flue-cured tobacco germplasm has narrow genetic diversity among the cultivars, necessitating a sustained effort to preserve flue-cured tobacco germplasm resources. Further crosses should be made only with genetically distant varieties.

Keywords: AFLP analysis; AMOVA; Flue-cured tobacco; Genetic diversity; UPGMA.

INTRODUCTION

China is the world's largest producer and consumer of tobacco. Flue-cured tobacco (*Nicotiana tabacum* L.) is one of the most important commercial crops in the world. The results of genetic diversity study provide estimates on the level of genetic variation among diverse materials that can be used in germplasm management, varietal protection, and flue-cured tobacco improvement.

Morphological, karyotypical, and physiological characters have already been used to study the genetic diversity of flue-cured tobacco germplasm (Goodspeed, 1945; Zhang, 1994; Lei et al., 1997; Lu, 1997). However, morphological characters usually vary with environments. The number of karyotypical characters is limited, and the study of genotypic diversity based on isozyme variation is restricted to a few polymorphic enzyme systems encoded by a small number of loci (Lu, 1997). These methods have been improved greatly by new molecular marker techniques such as simple sequence length polymorphism (SSLP), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), sequence characterized amplified regions (SCAR), and amplified fragment length polymorphism (AFLP) (Botstein et al., 1980; Jarman and Wells, 1989; Williams et al., 1990; Williams et al., 1991; Vos et al., 1995).

The AFLP technique allows the identification of a greater number of polymorphisms than RFLP or RAPD analysis. This technique is easy to perform compared to RFLP, reproducible, and requires only small amounts of DNA. Furthermore, it is a reliable and efficient DNA marker system that has been extensively used for genetic diversity study in different plant species (Maughan et al., 1996; Ellis et al., 1997; Breyne et al., 1999; Erschadi et al., 2000; De Riek et al., 2001). We employed the Amplified Fragment Length DNA Polymorphism (AFLP) technique to clarify the genetic relationships between 51 distinct flue-cured tobacco accessions with desirable agronomic characteristics from the germplasm collections of the South China Tobacco Breeding Research Center.

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MATERIALS AND METHODS

Plant materials

Seeds of 51 accessions of tobacco were obtained from the germplasm collections of the South China Tobacco Breeding Research Center in Yunnan Province, in southwestern China. The collection consists of 772 accessions from China and 12 foreign countries. On the basis of results from field trials conducted at Yuxi, Yunnan, from 1994 to 1996 (Lei et al., 1997), 51 accessions, were mainly from China and America, with desirable agronomic characteristics such as high leaf yield, low nicotine content, and resistance to various diseases or insects for this study. These accessions represented genotypes likely to be used in future flue-cured tobacco breeding efforts in south China. The names and origin of the cultivars are shown in Table 1.

Seeds were planted in pots in a greenhouse at temperatures of 28 to 32°C. Twenty days after germination, shoots were harvested from 40 seedlings of each accession representing the cultivar.

DNA extraction

DNA was extracted from shoots by the CTAB method (De Riek et al., 2001). DNA concentration and quality was estimated spectrophotometrically by measuring absorbance at 260 nm and by visual comparison with λDNA standards of known concentration on ethidium bromide (EtBr) stained agarose gels. DNA samples were diluted in TE-buffer and maintained at -20°C.

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. Restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters. Primers with *Eco*RI set included the sequence 5′- GAC TGC GTA CCA ATT C and the primers of *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 (2 μL) of loading dye (95% v/v formamide and 0.08% w/v bromophenol blue in 20 mM EDTA) was added to each sample, which was then denatured at 95°C for 3 min and placed on ice for 2 min before loading. Amplification products were analyzed by 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 (Fritz et al., 1999).

Data analysis

Each accession was scored 1 for presence or 0 for the absence of a band. Only bright, clearly distinguishable bands were used in the genetic analysis. Pairwise similarity matrices were generated using Jaccard's coefficient of similarity. A dendrogram was generated with the unweighted pair-group method with arithmetic average (UPGMA) algorithm as described by Sneath and Sokal (1973). The distance coefficient used for analysis was Nei's coefficient. The procedures above were performed using NTSYS-pc, Version 2.1. AMOVA and Fst calculations were performed with the software package Arlequin (Schneider et al., 2000).

RESULTS AND DISCUSSION

Twenty-two selective primer pairs were screened against all 51 accessions. Four primer pairs were not included in the final analysis because either the amplification profile was consistently too faint to score accurately (E-AAC/M-CGC) or only monomorphic amplification products were produced (E-ACG/M-CTG, E-AGC/M-CTC, E-ACT/M-CTG). The eighteen informative primer pairs used in the final analysis were listed in Table 2. Two (E-AAC+M-CTA) to thirty-two polymophic bands (E-ACA+M-CTA) of variable lengths were detected (Table 2), and the polymorphic rate ranged from 2.56% (E-AAC+M-CTA) to 37.10% (E-ACG+M-CAA). Three primer pairs were smaller than 10%; nine primer pairs ranged from 10% to 20%; three primer pairs

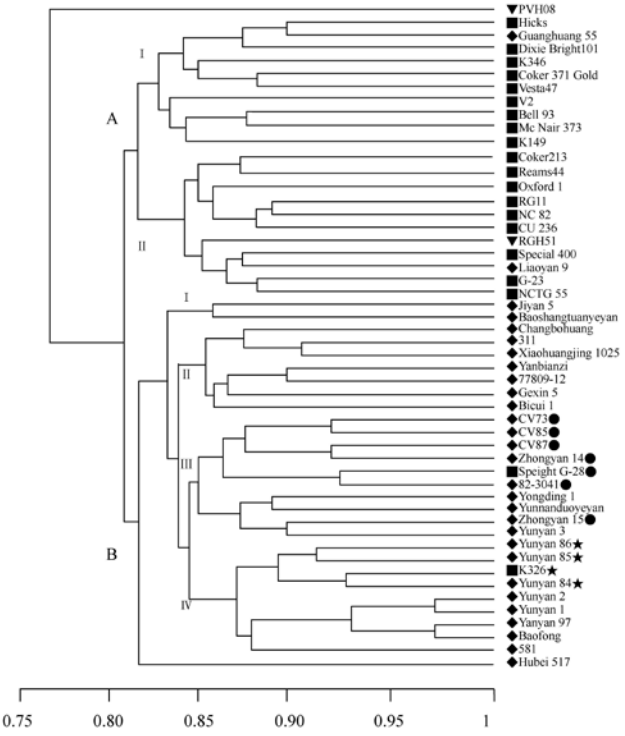

Figure 1. Dendrogram of the flue-cured tobacco cultivars using AFLP analysis. Symbols indicate country of origin: ◆ = China; ■ = USA; ▼ = Brazil. Symbols indicate ancestry of origin: ● = Speight G-28; ★=K326.

Table 1. 51 Flue-cured tobacco cultivars used in AFLP analysis.

No.	Cultivar	Pedigree	Origin
1	Hubei 517	NC2326	China
2	581	Chujingyan	China
3	Yunyan 3	Zhaojie 8 dui×Coker347	China
4	Zhongyan 15	Danyu 2×Speight G-28	China
5	Yunnanduoyeyan	Dajingyuan	China
6	Yongding 1	Special 401	China
7	Baofong	(401-2×G-80)×G-80	China
8	Yanyan 97	(401-2×G-80)×G-80	China
9	82-3041	G-28×Burley599	China
10	Zhongyan 14	Jingxing6007×Speight G-28	China
11	CV85	(CV58×Speight G-28)×[CV58×(G-28×NC82)F1]	China
12	Yunyan 84	Yunyan 2×K326	China
13	Yunyan 85	Yunyan 2×K326	China
14	Bicui 1	401	China
15	Gexin 5	Dahuangjing	China
16	77809-12	(Lingi 1×Virginia 115)F6	China
17	Yanbianzi	Unknown	China
18	Xiaohuangjing 1025	Xiaohuangjing	China
19	311	Yunyan 4×K329	China
20	Changbohuang	Unknown	China
21	Baoshangtuanyeyan	Unknown	China
22	Jiyan 5	Jingyehuang×Coker86	China
23	NCTG 55	K326×Coker 371-Gold	America
24	G-23	Unknown	America
25	Liaoyan 9	(5203×Ky 56)F1×(5637×Beiyu 29)F2	China
26	Special 400	Orinoco	America
27	RGH51	Unknown	Brazil
28	PVH08	Unknown	Brazil
29	CU 236	[(MC944×TI170) ×MC944]×K326	America
30	NC 82	6129×Coker319	America
31	RG11	NC50×K399	America
32	Oxford 1	[(Florida 301×Virginia Bright Leaf) ×Virginia Bright Leaf] ×Virginia Bright Leaf	America
33	Reams44	Coker319×Hicks	America
34	Coker213	Coker319×Coker139	America
35	K149	[G-28×Coker254]×(CB139×F-105)×(G-28×Coker254)]× McN399	America
36	Mc Nair 373	(Coker319×Coker139) × Mc Nair30	America
37	Bell 93	Bell 15×Coker 187-Hicks	America
38	V2	Unknown	America
39	Vesta47	Oxford×Yellow Special	America
40	Coker 371 Gold	[(G-28×354)×(CB139×F-105)×(G-28×354)]×NC82	America
41	K346	K326×80241	America
42	Dixie Bright101	[(TI448A×400)F3×Oxford]×(Florida301×4008)	America
43	Guanghuang 55	Jingxing6007×Dixie Bright 101	China
44	Hicks	White Stem Orinoco	America
45	Yunyan 1	Gold Dollar	China
46	Yunyan 2	Red Flowers Gold Dollar×Speight G-28	China
47	Speight G-28	(Oxford 1-181×Coker 139)F4×NC95	America
48	CV87	(CV58×Speight G-28)×[CV58×(G-28×NC82)F1]	China
49	CV73	(CV58×Speight G-28)×[CV58×(G-28×NC82)F1]	China
50	K326	McNair 225×(McNair 30×NC95)	America
51	Yunyan 86	Yunyan 2×K326	China

Table 2. The number of bands and degree of polymorphism revealed by AFLP primer combinations.

Primer combinations	Total bands	Polymorphic bands	Polymorphic rate (%)
E-ACG+M-CAA	62	23	37.10
E-AAC+M-CTA	78	2	2.56
E-AGC+M-CAC	85	13	15.29
E-AGG+M-CAG	81	9	11.11
E-ACA+M-CTA	113	32	28.32
E-AAG+M-CTG	77	8	10.39
E-AGC+M-CAA	94	21	22.34
E-AGG+M-CTT	86	14	16.28
E-AGG+M-CTA	57	21	36.84
E-ACT+M-CAC	102	12	11.76
E-AAC+M-CTG	91	20	21.98
E-ACA+M-CAC	86	15	17.44
E-ACA+M-CTT	74	11	14.86
E-AAG+M-CTC	89	3	3.37
E-ACT+M-CTC	67	8	11.94
E-AAG+M-CAG	86	12	13.95
E-ACG+M-CTA	93	4	4.30
E-ACG+M-CAG	58	21	36.21
Total	1479	249	
Average	82.17	13.83	16.84

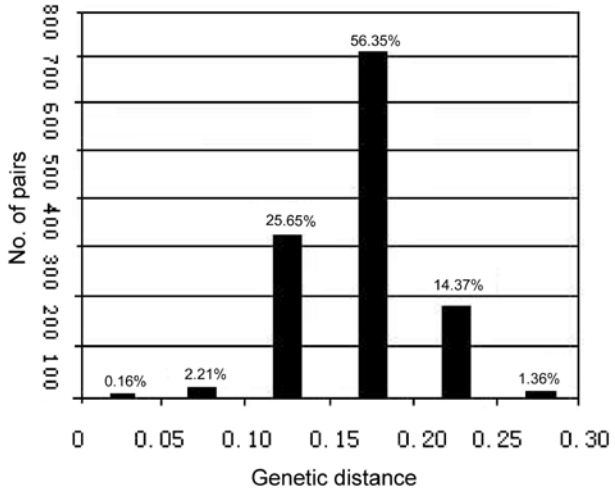

Figure 2. Distribution of pair-wise comparison of genetic similarity among tobacco cultivars.

ranged from 20% to 30%; and three primer pairs ranged from 30% to 40% (Table 2). Of course, the use of primer pairs selected for reproduction of higher polymorphism in the target group of genotypes could further increase the efficiency and the applications of the AFLP approach while the genetic loci which were invariant are also important for their potential to detect polymorphism in other flue-cured tobacco genotypes.

Fingerprinting revealed a total number of 1479 unambiguous DNA fragments with an average of 13.83 polymorphic loci per primer pair. The average polymorphism rate was 16.84%. Using these data, a dendrogram showing the relationships among the 51 accessions was created using NTSYS 2.1. Two distinct clusters were apparent in the dendrogram produced by cluster analysis (Figure 1). Group A consisted 21 accessions (41.18%), and group B included 29 accessions (56.86%). Interestingly, accession PVH08, a Brazilian cultivar, did not cluster with either of the groups.

The genetic variation among the 51 accessions was estimated using a pair-wise comparison of genetic distance. The average pairwise genetic distance was 0.167 and ranged from 0.024 to 0.267. 25.65% of them ranged from 0.10 to 0.15; 56.35% of them ranged from 0.15 to 0.20; and 14.37% of them ranged from 0.20 to 0.25. The results also showed that most of them (96.37%) ranged from 0.10 to 0.25, and only about 15.73% of the pair-wise comparisons among accessions exhibited a genetic distance greater than 0.20 (Figure 2). The most closely related cultivars, Yunyan 2, Yunyan 1, Yanyan 97, and Baofong, shared a genetic distance of 0.024. The least related, PVH08 and Bicui 1, had a genetic distance of 0.267. When the genetic variation of the cultivars was partitioned by AMOVA, 55.76% of the variation was found among the cultivars that had different geographic origins while 44.24% was found among the ones that had the same geographic origin. Both of the variation figures, within and among the origins, were highly significant ($P < 0.001$) (Table 3).

Mean Fst of each accession against the rest were shown in Figure 3. The overall average Fst of the 51 accessions was 0.177. In 29 Chinese cultivars, mean Fst of each accession against the rest ranged from 0.152 to 0.198, and the mean was 0.164. Those in American cultivars were 0.174 (mean), 0.152 (min.), and 0.193 (max.). In Brazil cultivars, they were 0.208 (mean), 0.178 (min.) and 0.238 (max.).The means were largest in Brazilian cultivars and smallest in Chinese cultivars. Low value of mean Fst revealed narrow genetic diversity, reflecting the consequence of inbreeding from a limited gene pool.

Table 3. AMOVA among and within geographic origins of the cultivars.

Source of variation	d.f.	Sum of squares	Variance components	Percentage	P-value
Among origin places	2	109.687	12.38099	55.76	<0.001
Within origin places	48	471.50016	9.82292	44.24	<0.001

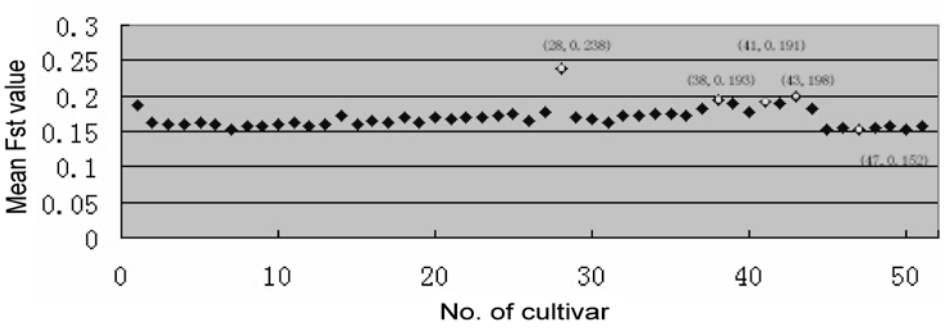

Figure 3. Mean Fst of each cultivar against the rest.

Characterization and quantification of genetic diversity has long been a major goal in breeding. In plant breeding programs, information on genetic diversity is essential for a rational use of genetic resources. It is particularly useful in characterizing individual accessions and cultivars, in detecting duplications of genetic materials in germplasm collections, and as a general guide in selecting parents for hybridisation in breeding programs and in developing informative mapping populations for genome mapping. Thus those more polymorphic primer pairs—such as E-ACG+M-CAA, E-AGC+ M-CAA, E-ACA+M-CTA, E-AGG+M-CTA and E-ACG+M-CAG—would be very useful in a flue-cured tobacco breeding program. In a study of wheat cultivars, Barrett and Kidwell (1998) detected 2-31 polymorphic bands per primer pair, with a mean polymorphic rate of 11.8%. Studies on rice cytoplasmic male-sterile (CMS) lines showed 8-23 polymorphic bands per primer pair, with an average of 16 polymorphisms (Subudhi et al., 1998). In Texas bluegrass genotypes Renganayaki et al. (2001) detected 3-116 polymorphisms per primer pair, with an average polymorphic rate of 64.11%. In this study, 2-32 polymophic bands per primer pair were detected, with an average polymorphic rate of 16.84%.

AMOVA results showed that most of the variations (55.76%) were found among the cultivars of different geographic origins. The dendrogram indicated a pattern of division among the flue-cured tobacco accessions based on geographic origin, as seen in some other crops (Spooner et al., 1996; Paul et al., 1997). The collection of American accessions was in cluster A, together with one Brazilian cultivar, RGH 51 and two Chinese cultivars. Chinese cultivars grouped together with two American cultivars and formed cluster B. The cultivars originated from different countries clustered together and shared the same ancestors. This can well be explained by the fact that lot of Chinese and Brazil cultivars were used to breed the American cultivars (Wang and Zhou, 1995). In general, Chinese cultivars clustered into four sub groups, and American cultivars clustered into two sub groups, with no clear pattern of division. Clearly, those shared common ancestry clustered together. For example, the cultivars bred by Speight G-28 such as 82-3041, Zhongyan 14, CV73, CV85 and CV87 clustered together in cluster BIII. Similarly, the cultivars, Yunyan 84, Yunyan 85 and Yunyan 86, sharing the same crossing parent K326, clustered together in cluster BIV.

Genetically more diverse genotypes may have good breeding value. PVH08, a Brazilian cultivar which did not cluster with any American or Chinese cultivars was the diverse one among all the genotypes used in the study. PVH08, V2, K346, and Guanghuang 55 were the genetically most distinct accessions. The mean Fst of these accessions was greater than 0.190, and these accessions would certainly contribute to the breeding program (Figure 3). Genotypes in the same cluster may represent members of one heterotic group. They displayed a similar DNA fingerprint. For example, Reams 44 and Coker 213 sharing common parent Coker 319 were clustered together in the cluster AII. A closer relationship was detected between the cultivars Baofong and Yanyan 97. Both cultivars have similar morphological traits and originated from the same parents (Lei et al., 1997). The results showed that AFLP assay could identify the flue-cure tobacco cultivars possessing similar genetic background. The availability of large numbers of polymorphic fragments enables the efficient evaluation of genetic diversity. The AFLP fingerprinting technique utilized in this paper has demonstrated that this powerful tool can be valuable in the breeding program of flue-cured tobacco.

Low level of pairwise genetic distance and mean Fst suggested the existence of limited genetic variation in flue-cured tobacco cultivars. The existence of low genetic diversity within cultivated flue-cured tobacco has been attributed to self-pollination (Wang and Zhou, 1995). A low DNA polymorphism level was also reported in several other self-pollinating plants such as wheat (Joshi and Nguyen, 1993), pigeonpea (Ratnaparkhe et al., 1995), tomato (Williams and St. Clair, 1993), and coffee (Steiger et al., 2002). It is also possible that a large proportion of valuable flue-cured tobacco germplasm may already have been lost through the popularity of certain cultivars in commercial planting and the continuous artificial selection. To avoid further degradation of germplasm resources, crosses should be made with genetically distant varieties or genotypes of diverse origin.

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應用擴增片段長度多型性研究烤煙遺傳歧異度

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烤煙是世界上最重要的商品作物之一。遺傳歧異度研究可對不同材料的遺傳變異水準進行估測，並可用於種質保存、品種保護，和烤煙品種的改良。對 51 個烤煙品種的擴增片段長度多型性 (AFLP) 分析產生了總數為 1479 條的明確的 DNA 片段。用非加權對組算術平均法 (UPGMA) 的聚類分析表明烤煙品種可劃分為美國型或中國型。中國型品種可進一步分為四個亞群，美國型品種也可進一步劃分為兩個亞群。品種間的平均成對遺傳距離為 0.167，變化範圍為從 0.024 到 0.267。AMOVA 分析表明 55.76% 的變異產生于不同起源地的品種間，44.24% 的變異則是來源於相同起源地的品種間。全部 51 個材料的遺傳分化指數 Fst 為 0.177，每個材料對其餘材料的平均遺傳分化指數 Fst 變化範圍在 0.152 到 0.238 之間。本研究的帶型所揭示的資訊表明，當今普遍種植的烤煙種質的遺傳歧異度很小，有必要採取長期有效的措施以保護烤煙種質資源，進一步的雜交也應當在遺傳關係較遠的品種間進行。

關鍵詞：擴增片段長度多型性分析；烤煙；遺傳歧異度；非加權對組算術平均法；分子變異分析。