PCR-RFLP Analysis of cpDNA in Tea Cultivars (*Camellia sinensis* L.) in Sichuan of China

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Received: November 16, 2011 Accepted: December 1, 2011 Online Published: April 1, 2012
doi:10.5539/jas.v4n5p25 URL: http://dx.doi.org/10.5539/jas.v4n5p25

The research is financed by Science & Technology Department Project of Sichuan Province (2006-YZGG-8).

Abstract

The genetic diversity among 30 tea cultivars in Sichuan of China was investigated by PCR-RFLP analysis of cp DNA. 7 sets of chloroplast primers could produce one or more than one distinct bands by direct electrophoresis in 2% agrose gels. After the amplified products were digested by 10 restriction enzymes, a total of 135 bands were detected, among which 98 bands (72.59%) were polymorphic. The cpDNA PCR-RFLP based genetic distance (GD) among 30 tea accessions ranged from 0 to 0.071, with the mean of 0.049.

Keywords: *Camellia sinensis*, PCR-RFLP, cpDNA

1. Introduction

A great number of genetic resources, including the tea plant and its allied species and varieties in genus *Camellia* have been collected and preserved in Sichuan Province, China. However, Cultivated tea is largely based on selection of yield, quality, biotic and abiotic stress resistance among the existing materials. As a consequence, the widspeard cultivation of clonal tea can diminish genetic diversity if care is not taken to use clones of disperse origin. So it appears necessary to collect and evaluate tea cultivars, which will provide important information on its phylogenetic relationship, and therefore, help to proper conservation and management of its genetic resources. Our preliminary investigations have shown a great deal of interspecific variation at the nuclear genome level (Wang *et al*. 2007). However, the organellar genome variations among them are not yet available.

The availability of universal primers capable of amplifying specific regions of the chloroplast (BADENES and PARFITT, 1995; TSUMURA et al., 1996; Heinze B, 2001) genomes using the polymerase chain reaction (PCR) has made it possible to explore organelle DNA diversity for taxonomic and phylogenetic purposes. Because of its uniparental mode of inheritance and its low mutation rate related to the nuclear genome, the chloroplast DNA (cpDNA) is considered to be an ideal system in phylogeny and in population genetics. Currently, sequence comparison or restriction analysis of fragments amplified with universal primers for organellar DNA has been widely used in species identification, genetic diversity and phylogenetic studies in many different plant species (GIELLY and TABERLET 1994; BADENES and PARFITT et al. 1995; DEMESURE et al. 1996; TSUMURA et al. 1996; PARDUCCI and SZMIDT 1999; HUANG and SUN 2000; PARANI et al. 2000, 2001; WANG et al. 2000; XU et al. 2001; WU et al. 2005).

The objective of this study was to evaluate the interspecific organelle genome variations in tea cultivars using restriction-site polymorphism of cpDNA, and to provide some more molecular data for phylogenetic relationships in *Camellia sinensis*.

2. Materials and Methods

2.1 Plant Material

The whole plant of different accessions were collected from Sichuan, Zhejiang, Fujiang, Hunan, Guangdong and Hainan provinces in China. 30 tea cultivars were transferred to the Tea Plant Garden of Sichuan Agricultural

University in Ya'an of Sichuan province, China. The accession numbers, origins and chromosome numbers are presented in Table 1.

2.2 DNA Extraction

Total genomic DNA was extracted from young leaves following the procedure of CTAB described by Huang (2003) with minor modifications.

2.3 RCR-RFLP Analysis

Seven sets of chloroplast primers were chosen for this investigation. Primer sequences are listed in Table 2. All the primers were synthesized by Shanghai Bioengineering Company. PCR amplification was performed in a 25μL reaction volume, containing 100ng template DNA, 0.2mmolL^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 50ng primer, 1×PCR buffer and 3U Taq DNA polymerase, covered with a drop of mineral oil. Amplification was performed in a PTC-220 Thermalcycler. Initial denaturation was for 3 min at 94℃, followed by 40 cycles of 1 min at 94℃, 1 min at 55℃, 3 min at 72℃ and a 10 min final extension step at 72℃. The success of each PCR reaction was verified by electrophoresis of 2l of the reaction products on 2% agarose gels in 1×TAE buffer and stained with ethidium bromide. The PCR-amplified DNA fragments were digested with the restriction endonucleases *Hinf* Ⅰ, *Hae* Ⅲ, *Hind* Ⅲ, *Taq* Ⅰ, *Msp* Ⅰ, *EcoR* Ⅰ, *Ssp* Ⅰ, *Rsa* Ⅰ, *Xba* Ⅰ,or *EcoR* Ⅴat 37℃ for 6 h. The digested DNA fragments were separated by electrophoresis on 2% agarose gels in 1×TAE and stained with ethidium bromide. Images were photographed, captured using ImageMaster VDS (Amersham PharmaciaBiotech).

2.4 Data Analysis

The digested DNA fragments were scored by presence (1) or absence (0) for each *C.sinensis* accession. Genetics similarities (GS) between each pair of accessions were estimated using the method of NEI and LI (1979), $GS=2N_{XY}/(N_X+N_Y)$, GD=1-GS, where N_X and N_Y are the numbers of DNA fragments observed in accession X and Y, respectively, and N_{XY} is the number of fragments shared by both accessions. All procedures were computed with the computer package NTSYS (ROHLF, 1993).

3. Results

3.1 PCR-RFLP Polymorphisms

All seven primers used in the present study successfully amplified the corresponding cpDNA regions in all the tea accession investigated. Digestion of the amplified products with *Hinf* Ⅰ, *Hae* Ⅲ, *Hind* Ⅲ, *Taq* Ⅰ, *Msp* Ⅰ, *EcoR* Ⅰ, *Ssp* Ⅰ, *Rsa* Ⅰ, *Xba* Ⅰ, or *EcoR* Ⅴ totally detected 135 fragments (Table 3), of which, 98 fragments (72.59%) were polymorphic. Fig. 1A illustrated the example of amplified products with primer trnL-trnF. Fig. 1B showed the digested products of trnL-trnF/Taq Ⅰ combinations.

3.2 Distances between Tea Cultivars

The genetic distances (GD) values between 30 tea accessions are presented in Table 4. The GD values among tea accessions varied from 0 to 0.071, with the mean of 0.049. The GD value between Zhe'nong113 and Zhe'nong117, Yingshuang and Jingfeng, Yinghong1 and Yinghong2, Mengshan 9 and Mengshan11 and mengshan23, was found to be the lowest (0). Zhe'nong113 and Zhe'nong117, Yingshuang and Jingfeng have lowest distances. This is because earlier two cultivars were the offspring of the same parents whereas later two have a common ancestral origin. And Yinghong1 and Yinghong2, Mengshan 9 and Mengshan11 and mengshan23, have lowest distances. This may be due to the reason that both the cultivars originated from a single clone (Bai, 2001).

4. Discussion

Interspecific variation could be detected through restriction analysis of fragments amplified with cpDNA universal primers (ZIEGENHAGEN et al. 1995; PARANI et al. 2001). This study showed that amplification of cpDNA with universal primers, followed by electrophoresis of restricted amplified fragments could reveal the interspecific polymorphism as 72.59%. Our previous study showed that the polymorphism of tea cultivars grown in Sichuan was high as 94.61% at the nuclear genome level (Wang et al. 2007). The genetic Distances (GD) of 30 Sicaun tea cultivara ranged from 0 to 0.071, and averaged at 0.049. That of tea cultivars grown in Sichuan ranged from 0.149 to 0.679, averaging at 0.412 (Wang et al. 2007). These suggested that relatively higher level of genetic polymorphism in tea cultivar could be detected by at the nuclear genome level, whereas relatively lower level genetic polymorphism could be estimated by cpDNA PCR-RFLP markers. This is in agreement with the results of investigations on *Cym bidium* (Gan et al. 2007).The reason why the genetic diversity of this study was lower that we can speculate that the chloroplast DNA (cpDNA) is uniparental mode of inheritance and its low mutation rate related to the nuclear genome in tea cultivars.

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Table 1. The accession name and source of tea cultivars

No	Accession Name	species	source	No	Accession Name	species	source
1	Mengshan 9	*C.sinensis*	Sichuan	16	Fuding	*C.sinensis*	Fujiang
2	Mengshan 23	*C.sinensis*	Sichuan	17	Yuanxiaocha	*C.sinensis*	Fujiang
3	Mengshan11	*C.sinensis*	Sichuan	18	Wuniuzao	*C.sinensis*	Zhejiang
4	Longjing 43	*C.sinensis*	Zhejiang	19	Zhe'nong117	*C.sinensis*	Zhejiang
5	Yingshuang	*C.sinensis*	Zhejiang	20	Donghuzao	*C.sinensis*	Hunan
6	Fuxuan 9	*C.sinensis*	Fujiang	21	Zhehedabaicha	*C.sinensis*	Fujiang
7	Anjibaicha	*C.sinensis*	Zhejiang	22	Fujiangshuixian	*C.sinensis*	Fujiang
8	Chunbolv	*C.sinensis*	Fujiang	23	Huangyeshuixian	*C.sinensis*	Guangdong
9	Meizhan	*C.sinensis*	Fujiang	24	Shuyong 307	*C.sinensis*	Sichuan
10	Zhuyeqi	*C.sinensis*	Hunan	25	Jingfeng	*C.sinensis*	Fujian
11	Fudingdahaocha	*C.sinensis*	Fujiang	26	Yinghong 1	*C.sinensis*	Guangdong
12	Juhuachun	*C.sinensis*	Zhejiang	27	Yinghong 2	*C.sinensis*	Guangdong
13	Longjingchangye	*C.sinensis*	Zhejiang	28	Qianmei 303	*C.sinensis*	Guizhong
14	Zhe'nong 113	*C.sinensis*	Zhejiang	29	Qianmei 419	*C.sinensis*	Guizhong
15	Pingyangtezao	*C.sinensis*	Zhejiang	30	Hainandaye	*C.sinensis*	Hannan

Table 2. DNA sequence and type of the primer pairs used in the present study

Prime rpair	Sequence	type	References
trnL-trnF	5′-CGAAATCGGTAGACGCTACG-3′ 5′-ATTTGAACTGGTGACACGAG-3′	cpDNA	TABERLET et al. 1991
trnT-trnL	5′-CATTACAAATGCGATGCTCT-3′ 5′-TCTACCGATTTCGCCATATC-3′	cpDNA	TABERLET et al. 1991
trnD-trnT	5′-ACCAATTGAACTACAATCCC-3′ 5′-CTACCACTGAGTTAAAAGGG-3′	cpDNA	DEMESURE et al. 1995
trnH-trnK	5′-ACGGGAATTGAACCCGCGCA-3′ 5′-CCGACTAGTTCCGGGTTCGA-3′	cpDNA	DEMESURE et al. 1995
trnS-trnfM	5′-GAGAGAGAGGGATTCGAACC-3′ 5'-CATAACCTTGAGGTCACGGG-3'	cpDNA	DEMESURE et al. 1995
rbcL	5′-TGTCACCAAAAACAGAGACT-3′ 5′-TTCCATACTTCACAAGCAGC-3′	cpDNA	PARANI et al. 2000
trnS-psbC	5′-GGTTCGAATCCCTCTCTCTC-3′ 5′-GGTCGTGACCAAGAAACCAC-3′	cpDNA	PARANI et al. 2000

Table 3. Amplified and digested DNA fragments of 30 tea accessions based on PCR-RFLP technology

Enzyme	*Hinf* Ⅰ		*Hae*Ⅲ		*Hind*Ⅲ		*Taq* Ⅰ		*Msp* Ⅰ		*EcoR* Ⅰ		*Ssp* Ⅰ		*Rsa* Ⅰ		*Xba* Ⅰ		*EcoR* Ⅴ	
Primer	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF	TF	PF
trnL-trnF	4	4	2	1	1	1	5	5	1	0	2	1	5	4	4	4	4	3	3	3
trnT-trnL					2	1			2	1									1	0
trnD-trnT	3	2	1	1	1	1	1	0			1	0								
trnH-trnK	5	3	2	2	1	1	1	0			1	0	2	1	1	1	4	3		
trnS-trnfM	3	3	2	2	1	1	1	1	1	0	1	0	2	2	1	1	5	4	1	0
rbcL	6	5	3	2	1	1	2	1	3	1	1	1	6	3	5	5	7	4	6	4
trnS-psbC	4	4	2	2	2	2			2	1			4	4	1	0			2	1

Note: TF:Total fragments;PF :Polymorphic fragments.

Table 4. The genetic Distances (GD) of 30 tea cultivars

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0.000																													
2	0.000	0.000																												
3	0.000	0.000	0.000																											
4	0.046	0.046	0.046	0.000																										
5	0.068	0.068	0.068	0.064	0.000																									
6	0.063	0.063	0.063	0.063	0.047	0.000																								
7	0.067	0.067	0.067	0.044	0.051	0.044	0.000																							
8	0.045	0.045	0.045	0.043	0.039	0.040	0.047	0.000																						
9	0.041	0.041	0.041	0.047	0.051	0.036	0.046	0.041	0.000																					
10	0.055	0.055	0.055	0.055	0.060	0.057	0.058	0.008	0.052	0.000																				
11	0.058	0.058	0.058	0.046	0.060	0.041	0.060	0.037	0.038	0.056	0.000																			
12	0.042	0.042	0.042	0.053	0.048	0.040	0.060	0.046	0.049	0.048	0.060	0.000																		
13	0.043	0.043	0.043	0.026	0.060	0.047	0.040	0.045	0.044	0.054	0.041	0.048	0.000																	
14	0.060	0.060	0.060	0.049	0.040	0.046	0.052	0.031	0.048	0.055	0.042	0.054	0.042	0.000																
15	0.049	0.049	0.049	0.042	0.052	0.047	0.051	0.040	0.042	0.062	0.048	0.051	0.038	0.043	0.000															
16	0.044	0.044	0.044	0.047	0.041	0.037	0.046	0.032	0.038	0.054	0.033	0.045	0.043	0.033	0.038	0.000														
17	0.047	0.047	0.047	0.046	0.050	0.047	0.042	0.038	0.032	0.062	0.032	0.053	0.041	0.057	0.052	0.035	0.000													
18	0.056	0.056	0.056	0.045	0.053	0.040	0.039	0.046	0.047	0.065	0.045	0.048	0.039	0.054	0.042	0.008	0.045	0.000												
19	0.060	0.060	0.060	0.049	0.040	0.046	0.052	0.031	0.048	0.055	0.042	0.054	0.042	0.053	0.043	0.033	0.038	0.054	0.000											
20	0.054	0.054	0.054	0.061	0.054	0.048	0.055	0.053	0.052	0.038	0.061	0.052	0.069	0.053	0.054	0.051	0.066	0.057	0.053	0.000										
21	0.045	0.045	0.045	0.042	0.048	0.034	0.041	0.032	0.000	0.060	0.035	0.050	0.038	0.047	0.043	0.034	0.029	0.042	0.047	0.051	0.000									
22	0.066	0.066	0.066	0.064	0.065	0.052	0.069	0.059	0.049	0.068	0.062	0.055	0.063	0.061	0.056	0.040	0.063	0.038	0.061	0.068	0.063	0.000								
23	0.056	0.056	0.056	0.055	0.063	0.058	0.062	0.054	0.063	0.069	0.062	0.054	0.064	0.063	0.057	0.038	0.065	0.038	0.060	0.046	0.055	0.069	0.000							
24	0.037	0.037	0.037	0.051	0.067	0.060	0.051	0.057	0.067	0.054	0.067	0.043	0.057	0.063	0.060	0.059	0.060	0.059	0.060	0.054	0.058	0.069	0.064	0.000						
25	0.058	0.058	0.058	0.064	0.060	0.057	0.051	0.067	0.062	0.035	0.066	0.049	0.060	0.060	0.062	0.041	0.060	0.053	0.060	0.062	0.048	0.064	0.063	0.058	0.000					
26	0.055	0.055	0.055	0.058	0.062	0.057	0.061	0.062	0.062	0.039	0.063	0.049	0.060	0.060	0.060	0.059	0.060	0.059	0.060	0.054	0.058	0.069	0.060	0.060	0.062	0.000				
27	0.065	0.065	0.065	0.068	0.062	0.057	0.061	0.062	0.062	0.039	0.063	0.049	0.060	0.060	0.060	0.059	0.060	0.059	0.060	0.054	0.058	0.069	0.060	0.060	0.062	0.000	0.000			
28	0.049	0.049	0.049	0.060	0.069	0.056	0.058	0.067	0.055	0.035	0.066	0.049	0.060	0.060	0.062	0.041	0.060	0.056	0.060	0.062	0.058	0.068	0.062	0.062	0.062	0.041	0.041	0.000		
29	0.037	0.037	0.037	0.051	0.055	0.048	0.043	0.058	0.051	0.054	0.068	0.045	0.051	0.057	0.060	0.054	0.053	0.048	0.057	0.050	0.049	0.070	0.060	0.025	0.062	0.068	0.068	0.054	0.000	
30	0.051	0.051	0.051	0.061	0.064	0.069	0.062	0.054	0.062	0.035	0.063	0.035	0.062	0.060	0.060	0.061	0.064	0.058	0.060	0.045	0.062	0.052	0.037	0.038	0.064	0.033	0.033	0.035	0.062	0.000

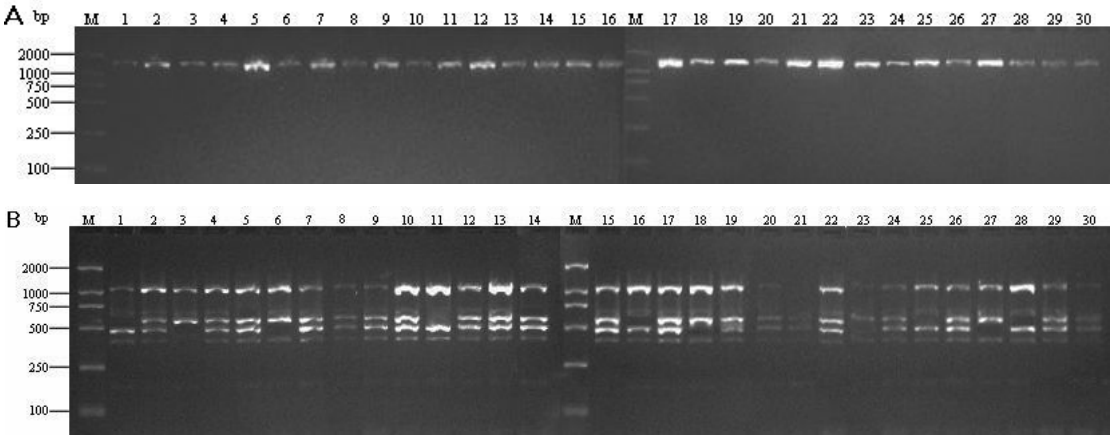

Figure 1. A-B. (A) Amplified products of primer pairs trnL-trnF of genomic DNA from 30 tea accessions. 1-30 indicate the number in Table 1. (B) Amplified and digested products of primer/enzyme combination trnL-trnF/Taq Ⅰ of genomic DNA from 30 tea accessions. 1-30 indicate the number in Table 1, M indicates DL2000 marker

ISSN 1916-9752 E-ISSN 1916-9760