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Full Length Research Paper

Rapid establishment of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) system for chloroplast DNA in tea [*Camellia sinensis* (L.) O. Kuntze]

Chen, S. X., Qi, G. N*., Li, H., Shan, H. L and Zou, Y.

Department of Tea Science, College of Horticulture, Sichuan Agricultural University, Ya'an 625014, China.

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A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) optimization reaction system for cpDNA in tea [*Camellia sinensis* (L.) O. Kuntze] was rapidly established. Results show that the optimal PCR reaction system was 100 ng template DNA, 200 μmolL^{-1} dNTPs, 1.5 mmolL^{-1} $MgCl_2$, 50 ng primer, 3U Taq DNA polymerase, and ddH_2O to the total volume of 25 μl; the optimal digestion system was 6 μl amplification product, 2 U endonuclease, 1×endonuclease buffer in digestion solution, and ddH_2O to the total volume of 15 μl; digestion time was 6 h at 37°C. With the optimized system, genetic diversity among 30 tea cultivars was investigated. Seven sets of chloroplast primers could produce one or more distinct bands. 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. This study suggests that the optimization system was suitable for PCR-RFLP analysis of cpDNA in tea.

Key words: *Camellia sinensis*, PCR-RFLP, chloroplast DNA, establishment.

INTRODUCTION

A great number of genetic resources, including tea and its allied species and varieties in the genus *Camellia*, have been collected and preserved in China. However, selection of cultivated tea is largely based on selection of yield, quality, biotic, and abiotic stress resistance among the existing materials. As a consequence, the widespread cultivation of clonal tea can diminish genetic diversity if care is not taken in the use of clones of disperse origin. So, it appears necessary to estimate the extent of genetic variation among tea cultivars, which may provide important information as to phylogenetic relationships. Having an understanding of genetic diversity may also provide insights as to proper conservation and management of its genetic resources. Several preliminary investigations have shown a great deal of interspecific variation at the nuclear genome level (Chen and Yamaguchi, 2005; Chen et al., 2005; Hung et al., 2007). However, the extent of variation among the organellar genome of tea plants is not yet known.

The availability of universal primers capable of amplifying specific regions of the chloroplast (Badens and Parfitt, 1995; Tsumura et al., 1996; Heinze, 2001) genome using 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, chloroplast DNA (cpDNA) is considered to be an ideal source of genetic information in phylogenetic and population genetic studies. 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

*Corresponding author. E-mail: guinian5612@sina.com. Tel: 86-835-288-2584.

Table 1. The name and source of tea cultivars.

Number	Cultivar name	Species	Source
1	Mengshan 9	*C. sinensis*	Sichuan
2	Mengshan 23	*C. sinensis*	Sichuan
3	Mengshan11	*C. sinensis*	Sichuan
4	Longjing 43	*C. sinensis*	Zhejiang
5	Yingshuang	*C. sinensis*	Zhejiang
6	Fuxuan 9	*C. sinensis*	Fujiang
7	Anjibaicha	*C. sinensis*	Zhejiang
8	Chunbolv	*C. sinensis*	Fujiang
9	Meizhan	*C. sinensis*	Fujiang
10	Zhuyeqi	*C. sinensis*	Hunan
11	Fudingdahaocha	*C. sinensis*	Fujiang
12	Juhuachun	*C. sinensis*	Zhejiang
13	Longjingchangye	*C. sinensis*	Zhejiang
14	Zhe'nong 113	*C. sinensis*	Zhejiang
15	Pingyangtezao	*C. sinensis*	Zhejiang
16	Fuding	*C. sinensis*	Fujiang
17	Yuanxiaocha	*C. sinensis*	Fujiang
18	Wuniuzao	*C. sinensis*	Zhejiang
19	Zhe'nong117	*C. sinensis*	Zhejiang
20	Donghuzao	*C. sinensis*	Hunan
21	Zhehedabaicha	*C. sinensis*	Fujiang
22	Fujiangshuixian	*C. sinensis*	Fujiang
23	Huangyeshuixian	*C. sinensis*	Guangdong
24	Shuyong 307	*C. sinensis*	Sichuan
25	Jingfeng	*C. sinensis*	Fujian
26	Yinghong 1	*C. sinensis*	Guangdong
27	Yinghong 2	*C. sinensis*	Guangdong
28	Qianmei 303	*C. sinensis*	Guizhong
29	Qianmei 419	*C. sinensis*	Guizhong
30	Hainandaye	*C. sinensis*	Hannan

species (Huang and Sun, 2000; Parani et al., 2001; Xu et al., 2001; Panda et al., 2003; Su, et al., 2005; Wei et al., 2005; Gan et al., 2006; Cui et al., 2006). The objective of this study was to perform optimization of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) system by orthogonal experiments and rapidly establish a PCR-RFLP reaction system for the analysis of cpDNA in tea. The study is to evaluate the genetic diversity of chloroplast genomes in cultivated tea, and provides some more molecular data for phylogenetic relationships in *Camellia sinensis*.

MATERIALS AND METHODS

Plant material and DNA extraction

The whole plant of 30 tea cultivars were collected from Sichuan, Zhejiang, Fujiang, Hunan, Guangdong and Hainan provinces in China and transferred to the Tea Plant Garden of Sichuan Agricultural University, Ya'an, Sichuan province, China. The cultivar names and origins are presented in Table 1. Total genomic DNA was extracted from young leaves following the CTAB procedure described by Huang (2003) with minor modifications.

Establishment and optimization of RFLP-PCR reaction system

Optimization of PCR reaction system

25 reaction systems were performed by the orthogonal experiment designed by $L_{25}(5^3)$ (Table 2). Template DNA and primer used in 25 reactions were from sample (Fuding) and primer trnL-trnF. All reaction volumes were 25 µl including 100 ng template DNA, 1.5 mmol L^{-1} $MgCl_2$, and 1×PCR buffer, covered with a drop of mineral oil. Amplification was performed in a PTC-220 Thermalcycler. Initial denaturation was for 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C and a 10 min final extension step at 72°C. Amplification products were verified by electrophoresis of 2µl of the reaction products on 2% agarose gels in 1×TAE buffer and stained with ethidium bromide for visualization.

Optimization of digestion system

16 digestion systems were performed by the orthogonal experiment

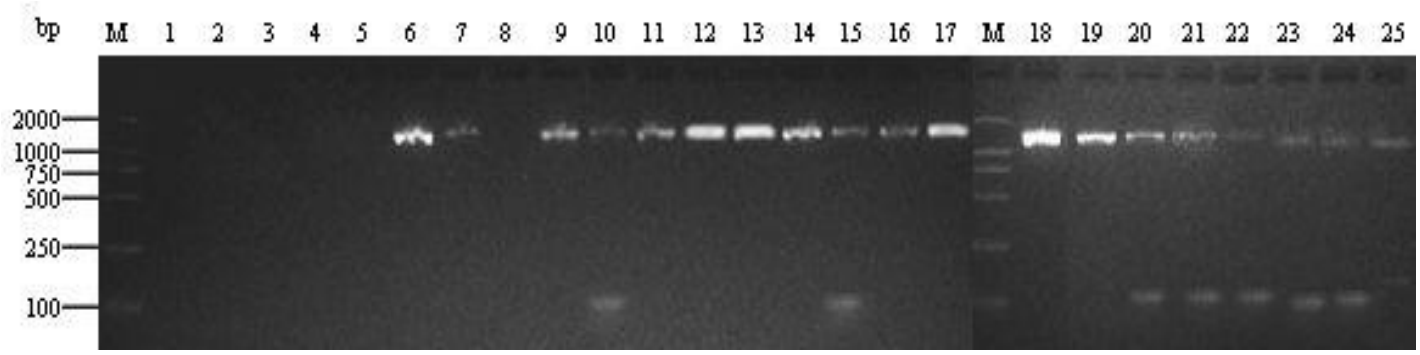

Figure 1. The results of L_{25} (5^3) orthogonal test (1-25 are orders listed in Table 2 and M is DL2000 marker).

designed by L_{16}(4^3) (Table 3). The PCR-amplified DNA fragments of Fuding with optimized PCR reaction system were digested with the restriction endonuclease *Taq* I. All reaction volumes were 15 µl including 1×endonuclease buffer in digestion solution, and sufficient quantum deionized H_2O, respectively. Digestion reactions were carried out at 37°C for 2, 6, or 8 h.

RCR-RFLP analysis

Seven sets of chloroplast primers were chosen for this investigation. Primer sequences are listed in Table 4. All the primers were synthesized by Shanghai Bioengineering Company. PCR amplification was performed with the aforementioned optimized PCR system. The PCR-amplified DNA fragments were digested by the restriction endonucleases *Hin*f I, *Hae III*, *Hin*d III, *Taq I*, *Msp I*, *Eco*R I, *Ssp I*, *Rsa I*, *Xba I* or *Eco*R V at 37°C with the afore stated optimized digestion system. The digested DNA fragments were separated by electrophoresis on 2% agarose gels in 1×TAE and stained with ethidium bromide. Images were photographed using ImageMaster VDS (Amersham PharmaciaBiotech).

Data analysis

The digested DNA fragments were scored by presence (1) or absence (0) for each *C. sinensis* accession. Genetic 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).

RESULTS AND DISCUSSION

Establishment and optimization of PCR - RFLP reaction system

With orthogonal experiments by L_{25} (5^3), all amplification products were analyzed by 2.0% agarose gel electrophoresis (Figure 1). Results show reaction system 12, 13, 17, 18 and 19 could amplify clear, stable bands. However, reaction system 12 cost the least in terms of amounts of reagents (Table 2). So, we believed that system 12 was a suitable, economic PCR reaction system for RFLP-PCR analysis on tea cultivars, that is, the optimization PCR reaction system was 100 ng template DNA, 200 µmolL^{-1} dNTPs, 1.5 mmolL^{-1} $MgCl_2$, 50 ng primer, 3U Taq DNA polymerase, and ddH_2O to the total volume of 25 µl.

16 digestion systems were performed by the orthogonal experiment designed by L_{16} (4^3) (Table 3). The PCR-amplified DNA fragments of Fuding with optimized PCR reaction system were digested with the restriction endonucleases *Taq I* (Figure 2). Results show that reaction system 8 could amplify clear, stable bands. So, we believed that system 8 was a suitable digestion reaction system for RFLP-PCR analysis on tea cultivars, that is, the optimization digestion reaction system was 6 µl amplification product, 2U endonuclease, 1×endonuclease buffer in digestion solution, and ddH_2O to the total volume of 15 ul; digestion time was 6 h.

PCR-RFLP polymorphisms and distances between tea cultivars

With the optimized system, all seven primers used in the present study successfully amplified the corresponding cpDNA regions in all the tea cultivars investigated. Digestion of the amplified products with *Hin*f I, *Hae* III, *Hin*d III, *Taq* I, *Msp I*, *Eco*R I, *Ssp I*, *Rsa I*, *Xba* I or *Eco*R totally detected 135 fragments (Table 5), of which, 98 fragments (72.59%) were polymorphic. Figure 3a illustrates the example of amplified products with primer trnL-trnF. Figure 3b shows the digested products of trnL-trnF/TaqI combinations.

The genetic distance (GD) values between 30 tea accessions are presented in Table 6. The GD values among tea accessions varied from 0 to 0.071, with the mean of 0.049. Fujiangshuixian and Huangyeshuixian have height GD of 0.071, while 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 had the lowest distances (0). This is

Table 2. Orthogonal design L_{25} (5^3) for PCR reaction system.

Order	Factor and level		
	Taq (U)	dNTP (μmolL^{-1})	primer (ng)
1	1	100	25
2	1	200	50
3	1	300	75
4	1	400	100
5	1	500	125
6	2	100	25
7	2	200	50
8	2	300	75
9	2	400	100
10	2	500	125
11	3	100	25
12	3	200	50
13	3	300	75
14	3	400	100
15	3	500	125
16	4	100	25
17	4	200	50
18	4	300	75
19	4	400	100
20	4	500	125
21	5	100	25
22	5	200	50
23	5	300	75
24	5	400	100
25	5	500	125

Table 3. Orthogonal design L_{16} (4^3) or digestion system.

Order	Factor		
	Amplification product (μL)	Restriction endonuclease (U)	Digestion time(h)
1	5	0.5	2
2	5	1	4
3	5	1.5	6
4	5	2	8
5	6	0.5	4
6	6	1	2
7	6	1.5	8
8	6	2	6
9	7	0.5	6
10	7	1	8
11	7	1.5	2
12	7	2	4
13	8	0.5	8
14	8	1	6
15	8	1.5	4
16	8	2	2

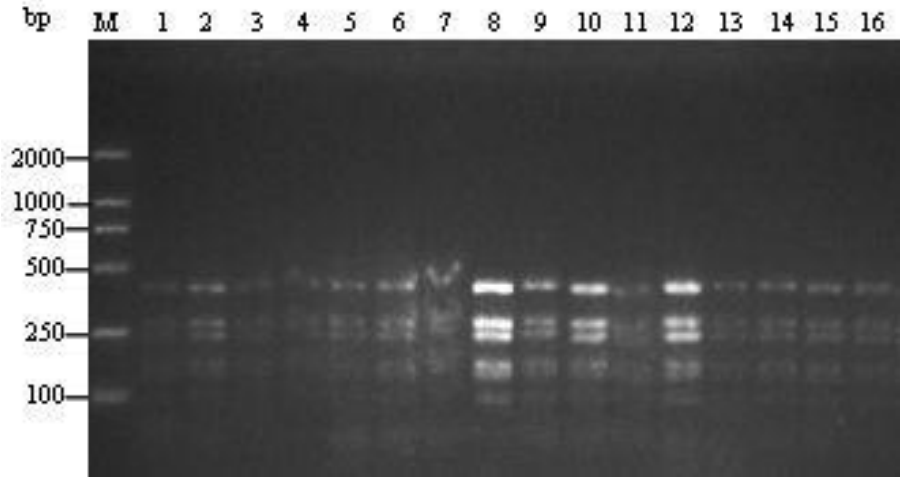

Figure 2. The results of L_{16} (4^3) orthogonal test (1-16) are orders listed in Table 3 and M is the DL2000 marker).

Table 4. DNA sequence and cpDNA primer pairs used in the present study.

Primer pair	Sequence	Reference
trnL-trnF	5´-CGAAATCGGTAGACGCTACG-3´ 5´-ATTTGAACTGGTGACACGAG-3´	Taberlet et al., 1991
trnT-trnL	5´-CATTACAAATGCGATGCTCT-3´ 5´-TCTACCGATTTCGCCATATC-3´	Taberlet et al., 1991
trnD-trnT	5´-ACCAATTGAACTACAATCCC-3´ 5´-CTACCACTGAGTTAAAAGGG-3´	Demesure et al., 1995
trnH-trnK	5´-ACGGGAATTGAACCCGCGCA-3´ 5´-CCGACTAGTTCCGGGTTCGA-3´	Demesure et al., 1995
trnS-trnfM	5´-GAGAGAGAGGGATTCGAACC-3´ 5'-CATAACCTTGAGGTCACGGG-3'	Demesure et al., 1995
rbcL	5´-TGTCACCAAAAACAGAGACT-3´ 5´-TTCCATACTTCACAAGCAGC-3´	Parani et al., 2000
trnS-psbC	5´-GGTTCGAATCCCTCTCTCTC-3´ 5´-GGTCGTGACCAAGAAACCAC-3´	Parani et al., 2000

because the earlier two cultivars were the offspring of the same parents whereas the later two had a common ancestral origin (Bai, 2001); while Yinghong1 and Yinghong2, Mengshan 9 and Mengshan11 and Mengshan23, have lowest distances (0).This may be due to the reason that both the cultivars originated from a single seed lot (Bai, 2001).

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 shows that under the optimized system, the amplification of cpDNA with universal primers followed by electrophoresis of restricted amplified fragments could reveal interspecific polymorphism, which was 72.59% among 30 tea cultivars in this study. An investigation on 15 Chinese elite tea genetic resources showed that the diversity was 94.2% (Chen et al., 2005). The diversity of 36 clonal tea cultivars in China was reported as 99.17% (Yao et al., 2007), 91.59% for 40 tea cultivars (Huang et al., 2006), and 91.89% for 43 tea cultivars (Tan et al.,

Table 5. Amplified and digested DNA fragments of the 30 tea accessions based on PCR-RFLP technology.

Enzyme	*Hinf* I		*Hae* III		*Hind* III		*Taq I*		*Msp I*		*Eco*R I		*Ssp I*		*Rsa I*		*Xba I*		*Eco*R 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

TF, total fragments; PF, polymorphic fragments.

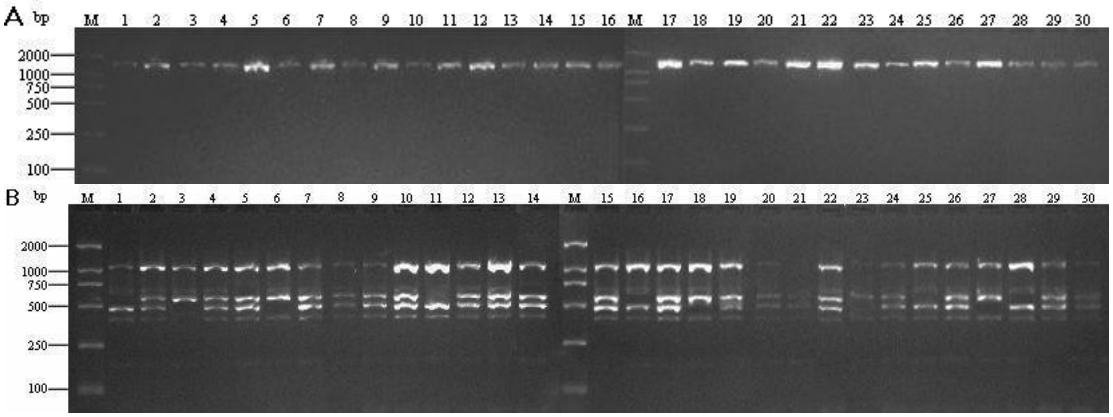

Figure 3. A-B. (A) Amplified products of primer pairs trnL-trnF of genomic DNA from 30 tea cultivars. 1-30 indicate the number in Table 1. (B) Amplified and digested products of primer/enzyme combination trnL-trnF/TaqI of genomic DNA from 30 tea accessions. 1-30 indicate the number in Table 1, M indicates DL2000 marker.

Table 6. The genetic distances (GD) of 30 tea cultivars based on PCR-RFLP technology.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
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	0.058	0.058	0.058	0.064	0.000										
6	0.053	0.053	0.053	0.053	0.047	0.000									
7	0.057	0.057	0.057	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	0.065	0.060	0.057	0.058	0.068	0.052	0.000					
11	0.058	0.058	0.058	0.046	0.050	0.041	0.050	0.037	0.038	0.066	0.000				
12	0.042	0.042	0.042	0.053	0.048	0.040	0.050	0.046	0.049	0.048	0.056	0.000			
13	0.043	0.043	0.043	0.026	0.060	0.047	0.040	0.045	0.044	0.064	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	0.065	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	0.064	0.033	0.045	0.043	0.033	0.038
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	0.067	0.052

18	0.056	0.056	0.056	0.045	0.053	0.040	0.039	0.046	0.047	0.059	0.045	0.048	0.039	0.054	0.042
19	0.060	0.060	0.060	0.049	0.040	0.046	0.052	0.031	0.048	0.065	0.042	0.054	0.042	0.000	0.043
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	0.059	0.053	0.054
21	0.045	0.045	0.045	0.042	0.048	0.034	0.041	0.032	0.030	0.060	0.035	0.051	0.038	0.047	0.043
22	0.066	0.066	0.066	0.064	0.065	0.052	0.059	0.059	0.068	0.069	0.056	0.055	0.063	0.061	0.056
23	0.056	0.056	0.056	0.065	0.063	0.058	0.062	0.054	0.063	0.049	0.062	0.054	0.064	0.056	0.057
24	0.037	0.037	0.037	0.051	0.057	0.050	0.063	0.057	0.058	0.054	0.067	0.043	0.057	0.063	0.056
25	0.058	0.058	0.058	0.064	0.000	0.047	0.051	0.039	0.051	0.060	0.050	0.048	0.060	0.040	0.052
26	0.055	0.055	0.055	0.058	0.062	0.057	0.061	0.062	0.052	0.039	0.063	0.049	0.054	0.060	0.061
27	0.055	0.055	0.055	0.058	0.062	0.057	0.061	0.062	0.052	0.039	0.063	0.049	0.054	0.060	0.061
28	0.049	0.049	0.049	0.060	0.059	0.056	0.058	0.067	0.055	0.035	0.066	0.049	0.065	0.067	0.064
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	0.050
30	0.051	0.051	0.051	0.061	0.064	0.059	0.062	0.064	0.062	0.035	0.063	0.055	0.062	0.060	0.060

Table 6. Contd.

	16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16	0.000														
17	0.035	0.000													
18	0.048	0.045	0.000												
19	0.033	0.038	0.054	0.000											
20	0.051	0.056	0.057	0.053	0.000										
21	0.034	0.029	0.042	0.047	0.051	0.000									
22	0.060	0.063	0.058	0.061	0.068	0.063	0.000								
23	0.058	0.065	0.058	0.056	0.046	0.055	0.066	0.000							
24	0.056	0.059	0.058	0.063	0.057	0.055	0.071	0.064	0.000						
25	0.041	0.050	0.053	0.040	0.054	0.048	0.064	0.063	0.058	0.000					
26	0.059	0.060	0.059	0.060	0.034	0.058	0.066	0.051	0.050	0.062	0.000				
27	0.059	0.060	0.059	0.060	0.034	0.058	0.066	0.051	0.050	0.062	0.000	0.000			
28	0.062	0.065	0.056	0.067	0.042	0.058	0.068	0.052	0.052	0.062	0.041	0.041	0.000		
29	0.054	0.053	0.048	0.057	0.050	0.049	0.070	0.050	0.025	0.055	0.048	0.048	0.054	0.000	
30	0.061	0.064	0.058	0.060	0.045	0.062	0.062	0.037	0.058	0.064	0.033	0.033	0.035	0.052	0.000

2009). The genetic distances (GD) of the 30 tea cultivars ranged from 0 to 0.071, and averaged at 0.049. The genetic distance of 15 Chinese elite tea genetic resources ranged from 0.16 to 0.62, and averaged at 0.37 (Chen et al., 2005). These suggest that relatively higher levels of genetic polymorphism in tea cultivars could be detected at

the nuclear genome level, whereas relatively lower levels of 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). Genetic diversity within the chloroplast genome may be lower than the nuclear genome because chloroplast DNA (cpDNA) is uniparentally inherited and has a lower mutation rate relative to the nuclear genome in most plants.

REFERENCES

Badens ML, Parfitt DE (1995). Phylogenetic relationships of cultivated *Prunus* species from an analysis of chloroplast DNA variation. Theor. Appl. Genet. 90: 1035-1041.

Bai FY (2001). China tea varieties. Shanghai: Shanghai science & technical publishers.

Chen L, Gao QK, Chen DM, Chen DM, Xu CJ (2005). The use of RAPD markers for detecting genetic diversity, relationship and molecular identification of Chinese elite tea genetic resources (*Camellia sinensis* (L.) O. Kuntze) preserved in tea germplasm repository. Biodiver. Conserv.14: 1433-1444.

Chen L, Yamaguchi S (2005). RAPD markers for discriminating tea germplasms at the inter-specific level in China. Plant Breed. 124: 404-409.

Cui BB, Li Y, Jin XJ, Feng H (2006). Genetic characters and polymorphism of chloroplastand mitochondrial DNA in white poplar. J. Beijing Forestry Univ. 28(6): 9-14.

Demesure B, Sodzi N, Petit RJ (1995). A set of universal primers for amplication of polymorphic noncoding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. 4: 129-131.

Gan N, Tan XH, Chen QB, Wei YM, Zheng YL (2006). Genetic Diversity in *Cymbidium* Based on RAPD Markers and PCR-RFLP Analyses of Organellar DNAs. Acta Horticulturae Sinica, 33(2): 349-355.

HEINZE B (2001). A data base for PCR primers in the chloroplast genome[DB]. http://bfw.ac.at/200/1859.html.

Huang JC, Sun M (2000). Genetic diversity and relationships of sweet potato and its wild relatives in Ipomoea series *Batatas* (Convolvulaceae) as revealed by intersimple sequence repeat (ISSR) and restriction analysis of chloroplast DNA. Theor. Appl. Genet. 100: 1050-1060.

Huang JA, Huang YH, Luo JW, Wang KB, Zhou LH (2003).Efficient methods for genomic DNA extraction from tea plant. J. Hunan Agric. Univ. 29(5): 403-407

Hung CY, Wang KH, Huang CC, Gong X, Ge XJ, Chiang TY(2008).Isolation and characterization of 11 microsatellite loci from Camellia sinensis in Taiwan using PCR-based isolation of microsatellite arrays (PIMA). Conserv. Genet. 9(3): 779-781.

Nei M, Li W (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl Acad. Sci. USA. 76: 5269-5273.

Panda S, Martin JP, Aguinagalde I (2003). Chloroplast DNA study in sweet cherry cultivars (*Prunus avium* L.) using PCR-RFLP method. Genet. Res. Crop Evol. 50: 489-495.

Parani M, Lakshmi M, Ziegenhagen B (2000). Molecular phylogeny of mangroves VII. PCR-RFLP of trnS-psbC and rbcL gene regions in 24 mangrove and mangrove-associate species. Theor. Appl. Genet. 100: 454-460.

Parani M, Rajesh K, Lakshmi M, Parducci L, Szmidt AE, Parida A (2001). Species identification in seven small millet species using polymerase chain reaction restrition fragment length polymorphism of trnS-psbC gene region. Genome, 44(3): 495-499.

Rohlf, FJ (1993). NTSYS-pc version 1.80. -/ Exeter Software, Setauket, NY.

Su YJ, Wang T, Zheng B, Jiang Y, Chen GP, Ouyang PY (2005). Genetic differentiation of relictual populations of Alsophila spinulosa in southenr China inferred from cpDNA trnL-F noncoding sequences. Mol. Phylogenet. Evol. 34: 323-333.

Taberlet P, Gielly L, Pauto G, Bouvet J (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17(5): 1105-1109.

Tsumura Y, Kawahara T, Wickneswari R, Yoshimura K (1996). Molecular phylogeny of Dipterocarpaceae in southeast Asia using RFLP of PCR-amplified chloroplast genes. Theor. Appl. Genet. 93: 22-29.

Xu DH, Abe J, Kanazawa A, Gai Y, Shimamoto (2001). Identification of sequence variations by PCR-RFLP and its application to the evaluation of cpDNA diversity in wild and cultivated soybeans. Theor. Appl. Genet. 102: 683-688.

Ziegenhagen B, Schauerte M, Scholz F, Kormutak A (1995). Restriction site polymorphism in chloroplast DNA of silver fir (Abies alba Mill.). For. Genet. 2: 99-107.

Yao MZ, Chen L, Wang XC, Zhao LP, Yang YJ(2007). Genetic diversity and relationship of clonal tea cultivars in China revealed by ISSR markers. Acta Agronomica Sinica, 33(4): 598-604.

Huang JP, Li JX, Huang YH, Luo JW, Gong ZH, Liu ZH(2006). Genetic Diversity of Tea (*Cam ellia sinensis* (L.) O.Kuntze) cultivars revealed by AFLP Ana lysis. Acta Horticulturae Sinica. 33(2): 317-322.

Tan YP, Li J, Liu SQ, Yan CY, Chen JH (2009). Genetic diversity of 43 tea cultivars (*Camellia sinensis*(L.) O. Kuntze) by SSR markers. J. Tea Sci. 29(4): 271-274.