

Full Length Research Paper

Optimization of RAPD-PCR reaction system for genetic relationships analysis of 15 camellia cultivars

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With orthogonal analysis by $L_{27}(3^6)$, the random amplified polymorphic DNA (RAPD)-PCR optimization reaction system for camellia were obtained. Results showed that the optimization system was 10×PCR Buffer (2.5 μL), 25 mM MgCl₂ (2.5 μL), 2.5 mM dNTPs (2.0 μL), 20 μM primer (1.0 μL), Tag (1.5 U), temple DNA (40 ng or so) and added ddH₂O to the total volume 25 uL; suitable annealing temperature was 36°C. With the optimized system and fifteen 10 nt random primers, we analyzed 15 camellia cultivars and observed 102 clear amplified loci, in which polymorphic loci were 79 while the percentage of polymorphic loci were 77.54%. Cluster analysis showed that the four groups were divided at the point 0.75 of similarity coefficient, indicating relatively high genetic diversity. We also found that the gene controlling petal color may play an important role in RAPD analysis. Moreover, genetic diversities based on RAPD analysis could be clearly reflected by morphological traits among 15 camellia cultivars. This study showed the RAPD optimization system was suitable and RAPD molecular marker was effective and useful tool for detection of genetic relationships among camellia cultivars.

Key words: Optimization, RAPD, Camellia cultivars, genetic relationships.

INTRODUCTION

Camellia features beautiful tree shape, various petal color, and graceful shape of flower. What is most important, it still blooms in winter during which few gardening plants could bloom. So camellia is regarded as an importantly ornamental horticultural plant. Moreover, it can afford raw material for tea beverage, edible oil and Chinese traditional herbal medicine (Ferrara et al., 2001; Chattopadhyay et al., 2004; Khan and Mukhtar, 2007; Gao et al., 2005; Tadahiro et al., 1985).

Genus Camellia belongs to *Theaceae* in *Theales*, grown natively in Southeast Asia. Presently, it has been introduced into many nonindigenous countries (Gao et al., 2005). Camellia is cross-pollinated plant and inter- and/or intra- species (or cultivars) hybrids naturally and/or artificially, frequently. Therefore, thousands of cultivars

have developed. It is estimated that about 15,000 camellia cultivars have been identified (Gao et al., 2005). Meanwhile, new cultivars and wild resources are still discovered frequently. This would bring much difficulty on identification, classification, molecular breeding and protection of wild resources. Therefore, it is very urgent and necessary to investigate camellia resources and explore their genetic relationships.

RAPD technology was firstly set up by Welsh and McClelland (1990) and Williams et al. (1990) at almost the same time. It could amplify genome DNA and investigate genetic diversities among samples with a set of random primers. Compared with other molecular markers, RAPD marker features sensitiveness, convenience and rapidness in detection, therefore, it was widely used in many fields such as analysis of genetic relationship (diversity), cultivars identification and gene localization in genome (Lima et al., 2007; Lin et al., 2009; Thiago et al., 2009; Ebrahimi et al., 2009). However, so far, we still know little genetic information on Camellia (or cultivars) because of limited reports by RAPD technology or by other molecular marker (Wachira et al., 1997; Kaundun et al., 2000, Kaundun and Young-Goo, 2002;

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Abbreviations: RAPD, Random amplified polymorphic DNA; PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphates; UPGMA, unweighed pair group method with arithmetic average.

Table 1. Code, Name and features of Camellia cultivars used in the study.

Code	Name	COF ¹	DOF ² (cm)	BP ³	OD ⁴
N1	Dujuancha (ND ⁵)	red	Middle	All the year	1.85
N2	Aomai (<i>C. nitidissima</i>)	Golden yellow	Small	Dec.-Feb.	1.45
N3	Jinsiyudie (<i>C. japonica</i>)	Bright white	Middle	Jan.-Mar	1.43
N4	Lianruicha (<i>C. Theopsis</i>)	white	Small	Mar.	1.45
N5	Liujiabai (<i>C. japonica</i>)	pure white	Middle	Feb.-Apr.	1.81
N6	Yumeiren (<i>C. reticulata</i>)	pure white	Large	Jan.-Mar	1.34
N7	Zuiyangfei (<i>C. japonica</i>)	bright red	Large	Feb-Mar.	1.43
N8	Fengxian (<i>C. japonica</i>)	pink	Small to middle	Mar.- Apr.	1.35
N9	Jiuzhonghua (<i>C. japonica</i>)	pink	Small	Feb-Mar.	1.33
N10	Hai'ermian (<i>C. japonica</i>)	pink	Large	Jan.-Mar.	1.59
N11	Moguangujing (from Japan, ND)	Dark red	Middle	Jan.-Mar.	1.38
N12	Hongluzhen (<i>C. japonica</i>)	red	Large	Jan-Mar.	1.45
N13	Qiumudan (<i>C. japonica</i>)	salmon pink	Large	Sep.-Dec.	1.41
N14	Fair Jury (from New Zealand, ND)	White dotted with violet red	Large	Jan-Mar.	1.39
N15	Star above star (from America, <i>C. sasanqua</i>)	White with pale purple	Large	Sep.-Dec	1.32

Camellia names from Shao and Gao (2003). COF¹: color of flower; DOF²: diameter of flower, that is, small (5 – 6 cm), middle (7 – 11 cm), large (12 – 14 cm); BP³: blooming period; OD⁴: A260/A280; ND⁵: not detected.

Tang et al., 2006; Xiao et al., 2008; Kunjupillai et al., 2009).

RAPD-PCR reaction system features tiny volume (25 μ L, generally), many ingredients (dNTP, polymerase, primers, template, etc) and many steps (pre-denaturing, denaturing, annealing, extension) and many cycles (30 - 40). So little change of any parameters could affect products drastically (Diakou and Dovas, 2001; Fraga et al., 2002), which indicates its kinetics profile very complex. Therefore, the RAPD-PCR reaction system must be optimized before its application on any kind of materials. The present study performed optimization of RAPD-PCR system by orthogonal experiments and investigated genetic relationships on 15 representative camellia cultivars. The study would do good to further investigation of camellia genome diversity, cultivars identification, classification and protection, molecular breeding, etc.

MATERIALS AND METHODS

Plant materials and their Genomic DNA extraction

All camellia cultivars were collected from Zhejiang Hongxin Garden Company (Wenzhou, China). Of them, one common cultivar (Huzhuabai (*C. japonica*)) was randomly selected for optimization of RAPD-PCR reaction system and other 15 representative cultivars (Table 1) were analyzed with optimized reaction system. Genomic DNA was isolated from camellia tender leaf tissue (in March) with hexa-adearyl-tri-methyl ammonium bromide method (Doyle and Doyle, 1987). The purity and quantity of genomic DNA were

detected by spectrophotometry (Thermo electron corp Biomate 5).

Establishment and optimization of RAPD-PCR reaction system

Optimization of reaction system: Twenty-seven reaction systems were performed by the orthogonal experiment designed by $L_{27}(3^6)$ (Table 2). Temple DNA and primer used in 27 reactions were from sample (Huzhuabai) and primer S7 (GGTGACGCAG) selected randomly. All reaction volumes were 25 μ L including 2.5 μ L 10 \times Buffer (without Mg^{2+}) and sufficient quantum deionized H_2O , respectively. PCR reaction was carried out in a thermal cycler (Tpersonal, Whatman Biometra), programmed for 1 cycle of 3 min at 94 $^{\circ}$ C for pre-denaturation, followed by 40 cycles of 94 $^{\circ}$ C for 30 s (denaturing), different temperature for 60 s (annealing) and 72 $^{\circ}$ C for 90 s (extension). Additional extension (72 $^{\circ}$ C for 10 min) was used to ensure the completeness of PCR products. Amplification products were stored at 4 $^{\circ}$ C for detection.

Analysis of 15 camellia cultivars by RAPD-PCR amplification

With fifteen 10 nt random primers (Table 3) (Shanghai Bioengineering Company, China), PCR amplification reactions were carried out with the above optimized system, respectively. All PCR products were separated by 1.2% agarose gel electrophoresis in 0.5 \times TBE buffer, then stained by ethidium bromide (EB) and visualized, photographed under UV light (Gel Doc-It Imaging system (UVP, Bio Doc Co., USA)). Figure 1 represented electrophoresis map from RAPD-PCR amplification reaction products by partial primers.

Data processing and cluster analysis

All gel bands were scored and the similarity (S) were analyzed and

Table 2. Orthogonal design $L_{27}(3^6)$ for RAPD-PCR analysis.

Treatment sequence	Factors and levels					
	MgCl ₂ (25 mM) (μL)	dNTP (each 25 mM) (μL)	Primer (20 μM) (μL)	Template (ng)	Tag (U)	Tm (°C)
1	1 (1.5)	1 (1.0)	1 (1.0)	1 (40)	1 (1.0)	1 (34)
2	1	1	1	1	2 (1.5)	2 (36)
3	1	1	1	1	3 (2.0)	3 (38)
4	1	2 (2.0)	2 (2.0)	2 (60)	1	1
5	1	2	2	2	2	2
6	1	2	2	2	3	3
7	1	3 (3.0)	3 (3.0)	3 (80)	1	1
8	1	3	3	3	2	2
9	1	3	3	3	3	3
10	2 (2.0)	1	2	3	1	2
11	2	1	2	3	2	3
12	2	1	2	3	3	1
13	2	2	3	2	1	2
14	2	2	3	2	2	3
15	2	2	3	2	3	1
16	2	3	1	1	1	2
17	2	3	1	1	2	3
18	2	3	1	1	3	1
19	3 (2.5)	1	3	2	1	3
20	3	1	3	2	2	2
21	3	1	3	2	3	1
22	3	2	1	1	1	3
23	3	2	1	1	2	2
24	3	2	1	1	3	1
25	3	3	2	3	1	3
26	3	3	2	3	2	2
27	3	3	2	3	3	1

calculated (Nei and Li, 1979; Rohlf, 2000). A dendrogram was constructed based on similarity matrix unweighed pair group method with arithmetic average cluster analysis (UPGMA) (Sneath and Sokal, 1973).

RESULTS

Orthogonal analysis on establishment and optimization of RAPD-PCR reaction system

With orthogonal experiments by $L_{27}(3^6)$, all amplification products were analyzed by 1.2% agarose gel electrophoresis. Results showed reaction system 23 (Table 2) could amplify clear, stable and reproducible bands, so we believed that system 23 was a suitable combination for RAPD analysis on camellia cultivars, that is, the optimi-

zation system was 10×PCR Buffer (2.5 μL), 25 mM MgCl₂ (2.5 μL), 2.5 mM dNTPs (2.0 μL), 20 μM primer (1.0 μL), Tag (1.5 U), temple DNA (40 ng or so) and additional ddH₂O to the total volume 25 μL, suitable annealing temperature was 36°C.

Analysis on genotype polymorphism among the 15 camellia cultivars

With the optimization system, 15 representative samples were analyzed by 15 primers, respectively, the results showed that the total of clear 102 loci were produced, 79 of which were of polymorphism, indicating the percentage of polymorphic site was 77.54%; The amplification sizes ranged from 0.3 to 4.0 kb or so. For similarity coefficient

Table 3. Primers and sequences and each amplification results from 15 samples.

Primer	Sequence	Total bands	Polymorphic bands	RPB ¹ (%)
S10	CTGCTGGGAC	7	7	100%
S11	GATGACCCGT	10	7	70.0%
S20	GGACCCTTAC	4	4	100%
S32	TCGGCGATAG	8	7	87.5%
S39	CAAACGTAGG	10	9	90.0%
S43	GTCGCCGTCA	8	6	75.0%
S112	ACGCGCATGT	6	5	83.3%
S128	GGGATATCGG	4	3	75.0%
S216	GGTGAACGCT	5	4	80.0%
S254	TGGGTCCCTC	4	2	50.0%
S256	CTGCGCTGGA	7	4	57.1%
S263	GTCCGGAGTG	6	4	66.7%
S265	GGCGGATAAG	7	4	57.1%
S267	CTGGACGTCA	8	6	75.0%
S300	AGCCGTGGAA	8	7	87.5%
Total	102	79	77.54%	
Average	6.8	5.27	77.50%	

RPB¹ stands for ratio of polymorphic bands (%).

Table 4. Genetic similarities among 15 samples.

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15
N1	1														
N2	0.632	1													
N3	0.611	0.693	1												
N4	0.673	0.653	0.714	10.											
N5	0.628	0.628	0.752	0.690	1										
N6	0.581	0.663	0.806	0.724	0.793	1									
N7	0.571	0.693	0.755	0.673	0.762	0.785	1								
N8	0.642	0.622	0.724	0.683	0.845	0.795	0.765	1							
N9	0.648	0.642	0.806	0.683	0.793	0.795	0.826	0.816	1						
N10	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					
N11	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					
N12	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					
N13	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					
N14	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					
N15	0.597	0.711	0.793	0.659	0.760	0.804	0.814	0.762	0.845	1					

N1	0.612	0.612	0.795	0.693	0.742	0.744	0.735	0.724	0.846	0.773	1					
1	2	2	9	9	3	9	0	5	9	2						
N1	0.591	0.653	0.775	0.673	0.762	0.744	0.795	0.704	0.785	0.845	0.795	1				
2	8	1	5	4	9	5	9	0	7	4	9					
N1	0.608	0.670	0.814	0.752	0.835	0.814	0.824	0.824	0.876	0.822	0.762	0.783	1			
3	2	1	4	6	0	4	7	7	3	9	9	5				
N1	0.648	0.659	0.755	0.734	0.784	0.744	0.776	0.744	0.787	0.784	0.819	0.787	0.838	1		
4	9	6	3	0	9	7	6	7	2	9	1	2	7			
N1	0.638	0.691	0.744	0.659	0.752	0.712	0.787	0.755	0.734	0.752	0.747	0.712	0.784	0.819	1	
5	3	5	7	6	7	8	2	3	0	7	7	8	9	1		

among the 15 samples, they widely ranged from 0.5714 to 0.8763 (Table 4), which represented the closest genetic relation between N₉ and N₁₃ and the furthest between N₁ and N₇, respectively.

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Cluster analysis

The Figure 2 represented the genetic relationships among 15 camellia cultivars. The 15 samples were distinctly

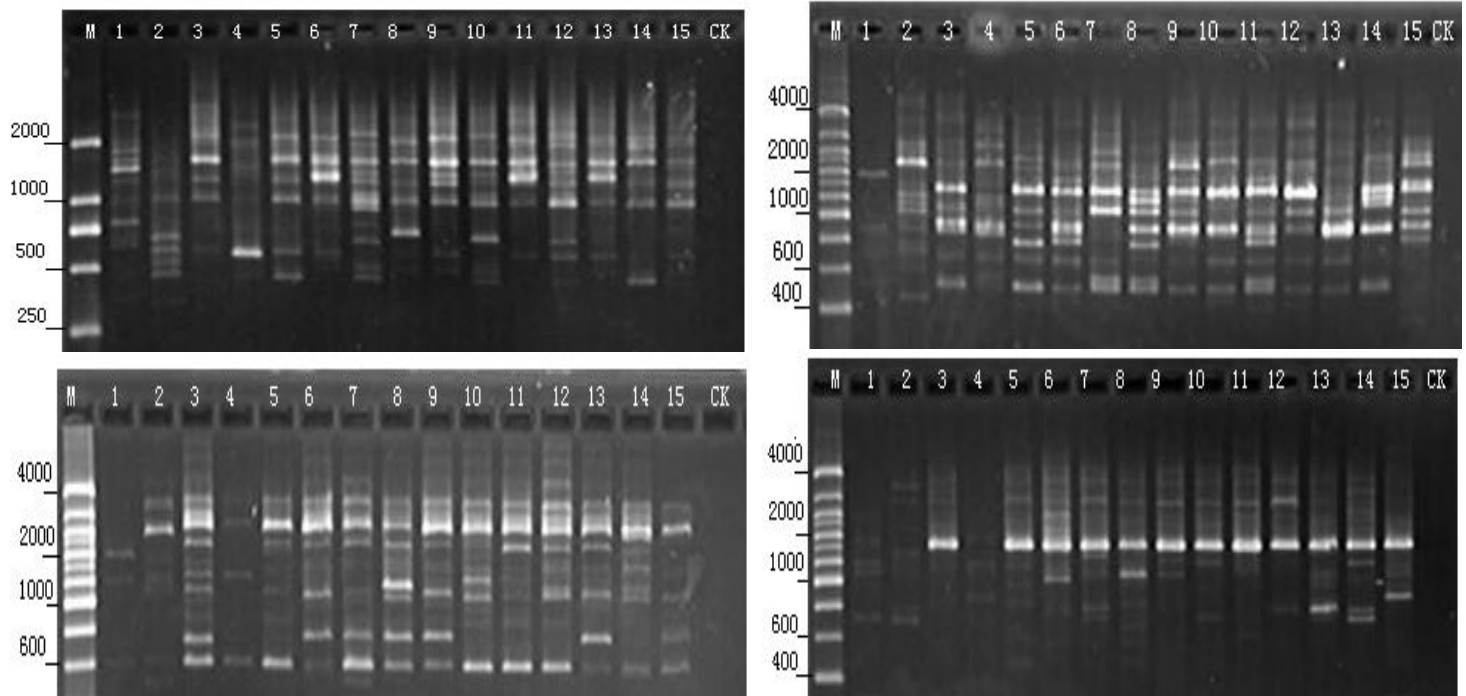


Figure 1. Agarose gel electrophoresis analysis by primer S256 (left-up), S39(left-down) and primerS267(right-up), primerS300(right-down),respectively. M stands for DNA Marker while CK was negative control. Samples from lane1-15 were the same to their serial number (Table 1).

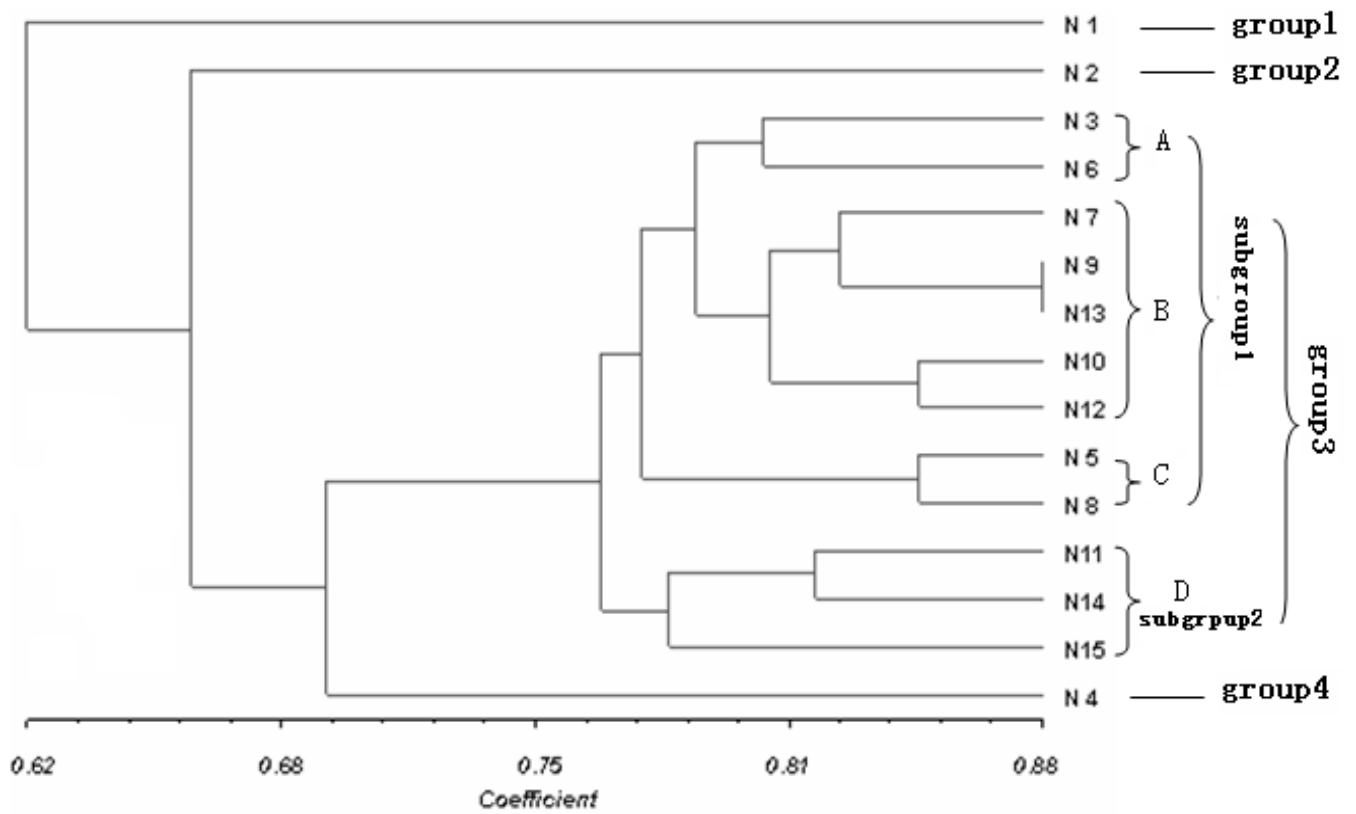


Figure 2. UPGMA dendrogram of 15 samples genotypes based on RAPD-PCR analysis by 15 random RAPD primers.



Figure 3. Morphological traits of two unique camellia varieties (left: Dujuanacha (N₁); right: Aomai (N₂)).

clustered into four groups at the point 0.75 of similarity coefficient, that is, N₁ (group 1); N₂ (group 2); N₃ and N₅₋₁₅ (group 3); N₄ (group 4). If we considered group 3 in detail, it could be furthered to divide into two subgroups

(Figure 2): Subgroup1 included A(N₃ and N₆)/ B(N₇,N₉,N₁₃,N₁₀,N₁₂)/ C(N₅and N₈) while subgroup 2 included D(N₁₁ and N₁₄, N₁₅).

DISCUSSION

The volume of RAPD-PCR system was so tiny that change of any ingredient concentrate would affect products drastically. Therefore, it was so difficult even impossible to investigate single ingredient effect (Diakou and Dovas, 2001; Fraga et al., 2002). The present study got suitable RAPD system by orthogonal analysis because of comprehensive consideration of all elements in RAPD analysis. It also indicated that Mg^{2+} concentrate and annealing temperature could bring big effect (Hernandez et al., 1999). In the study, the genome DNA was isolated from tender leaf tissue and was purified two times by phenol-chloroform after complete treatment of RNase. This resulted in little impurity (such as protein, RNA) in genome DNA and their ratios of A260/A280 ranged from 1.32 to 1.85 (Table 1), indicating available amplification effect. Theoretically, the DNA concentrate should be as low as possible because high concentrate DNA itself may bring false positive bands. The present study acquired ideal effect with 40 ng genome DNA.

The genetic diversities based on RAPD analysis could be reflected by morphological traits. For example, the leaf of sample N1 (Djuancha (*C. japonica*)) was thick, cerasaceous and smooth with red petals, moreover, it was a unique camellia species blooming all the year; For sample N2 (Aomai (*C. nitidissima*)), its petal was unique yellow and small, a kind of exceptional and cherished camellia cultivar (Figure 3); As for sample N₄ (Lianruicha (*C. Theopsis*)), it featured narrow and long leaf, small flower and soft branch; Other 12 samples were of similar morphological traits. In other words, samples N₁, N₂, N₄ showed great morphological differences one another compared with other 12 cultivars. Therefore, it was understandable that three samples (N₁, N₂, N₄) clustered into different groups, respectively, while other 12 samples clustered closely (Figure 2). The result also showed that RAPD marker was feasible and suitable to analyze the genetic diversities among camellia cultivars. Meanwhile, it also furthered to represent the RAPD optimization system in this study was suitable.

For group 3, an interesting thing was observed that samples in each subgroup featured similar morphological traits (Table 1). For the petal color of samples in Subgroup 1, N₃ and N₆ were similar (white); N₇, N₉, N₁₃, N₁₀, N₁₂ were similar (red); while N₅ (white) and N₈ (pink) had a little difference. These morphological differences could be clearly represented in the Figure 2, that is, sample N₃ and N₆ clustered closely (A); sample N₇, N₉, N₁₃, N₁₀, N₁₂ clustered closely (B) while sample N₅ and N₈ clustered together (C). In other words, after A and B clustered, they furthered to cluster with C. This reminded us that the gene(s), controlling petal color, could play critical roles in RAPD analysis. Of course, other genes, which controlled blooming period and flower diameter also played positive roles in RAPD analysis. Actually, the RAPD analysis was very complex, all genes would contribute to positive effect

though some genes play big roles while others play little roles. Another interesting thing was involved in three samples (N₁₁, N₁₄, N₁₅) in subgroup 2 (D). Though they were introduced to China from different countries (Table 1), they could cluster together closely. One possible explanation was three samples could be hybrid progeny of the same camellia species (or cultivar) and were introduced into different countries, respectively. Therefore, they had relatively high genetic similarities.

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