GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

RAPD-based genetic diversities and correlation with morphological traits in *Camellia* (Theaceae) cultivars in China

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Genet. Mol. Res. 10 (2): 849-859 (2011)
Received December 13, 2010
Accepted February 25, 2011
Published May 10, 2011
DOI 10.4238/vol10-2gmr1207

ABSTRACT. *Camellia* is an economically important ornamental plant that has many uses, such as in beverages, foods and medicines. We examined 15 *Camellia* cultivars in Wenzhou, China, using RAPD markers and measurements of three traits (petal color, flower diameter, blooming period). PCR amplification with 15 random primers produced 1935 bands, observed at 88 amplification loci; 77% of the amplified loci were polymorphic, with a mean of 4.5 polymorphic loci per primer. The similarity coefficient ranged from 0.5419 to 0.7933 among the 15 samples; the lowest value was between Manao (*C. reticulata*) and Feibai FR (*C. japonica*), and the largest value was between Chidan (*C. japonica*) and Yuanyang FG (*C. japonica*). Cluster analysis divided the 15 cultivars into two groups at the similarity coefficient of 0.65. A correlation was found between RAPD markers and petal color in the first group. No correlation was found between RAPD markers and the other traits (flower diameter, blooming period). This study provides information useful for the identification, classification, phylogenesis, and breeding of *Camellia* cultivars.

Key words: *Camellia*; Genetic diversity; Correlation; Petal color; Morphological trait; RAPD marker

INTRODUCTION

Camellia is an economically important ornamental plant throughout the warm temperate zones, which belongs to the genus *Camellia* in the family Theaceae (Gao et al., 2005). It can grow well under semi-shaded conditions with wet-warm climate, and features an evergreen appearance, diverse flower colors (red, white, yellow, etc.), graceful flower shapes, and a long blooming period (4-6 months, or even all year) (Gao et al., 2005). Notably, it can bloom in late winter, whereas most plants fail to do so during that time. Additionally, it contains rich volatile oils, vitamins, minerals, antioxidants, and polyphenols. Therefore, it is often used as a raw material for tea beverages, edible oils, spices, traditional herbal medicine, and cosmetic candidates (Ferrara et al., 2001; Kim et al., 2001; Gao et al., 2005; Khan and Mukhtar, 2007; Jung et al., 2007).

So far, over 15,000 *Camellia* cultivars have been identified and documented in horticulture, which mainly belong to *C. japonica*, *C. reticulata*, *C. sasanqua*, and *C. nitidissima* (Gao et al., 2005), and new *Camellia* cultivars still gradually occur as a result of frequent hybridization and artificial selection under cultivation or natural surrounding. Notably, almost all *Camellia* cultivars are named according to morphological traits such as flower shape and color, and tree or leaf shape. Therefore, disagreement on or discrepancy in the classification of *Camellia* cultivars inevitably occurs occasionally. On the one hand, it is very difficult to distinguish extremely similar morphological traits such as flower color or shape, and different investigators may draw different conclusions on the same cultivar. On the other hand, some cultivars that have a relatively close genetic relationship show a relatively large morphological difference due to growing changes in surroundings. Comparatively, genomic DNA represents the complete genetic information of species (or cultivars) and is not affected by surroundings, climate and developmental phase. Therefore, molecular techniques are more objective and make it possible to identify, classify or characterize genetic relationships between samples (Xiao and Clifford, 2003).

Presently, the characterization of plant genetic diversities or relationships is mainly performed based on polymerase chain reaction (PCR) amplification or sequence analysis approaches, including RFLP (restriction fragment length polymorphism), ISSR (inter-simple sequence repeat), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and SNP (single nucleotide polymorphisms) (Powell et al., 1996; Khlestkina and Salina, 2006). Recently, new approaches such as nrITS (nuclear ribosome internal transcribed spacer) (Vijayan et al., 2009) and DNA barcode (such as plastid DNA sequence) (Kress et al., 2005) are popularly applied to analyze genetic diversities and relationships. Generally, each approach features its particular sensitivity and applicability. In comparison, RAPD analysis could cover the whole genome information, and is still a convenient, effective and low-cost technology, which has been widely and successfully applied to explore genetic relationships (Dorokhov and Klocke, 1997; Ahlawat et al., 2010; Leal et al., 2010). Briefly, it could amplify the whole genomic DNA by the PCR technique with a set of 10-nt random primers with each primer having a specific binding site(s) in genomic DNA. Therefore, the information of amplification products by all primers could disclose genetic diversities among samples.

Camellia features rich genetic diversities (Chung and Kang, 1996). Ueno et al. (1999, 2002) investigated the genetic structure and spatial distribution of individuals within a population of *C. japonica*, who disclosed high diversities and no significant differences in allele frequencies among different size-classes. Xiao and Clifford (2003) identified the genus *Camellia* by analyzing DNA sequences of the RPB-2 gene (nuclear RNA polymerase II), and provided

strong support for the hypothesis that the genus *Camellia* is a monophyletic group. However, they obtained conflicting results concerning partial sub-generic divisions and debated sections of the genus *Camellia* compared with previous studies. Tang et al. (2006) analyzed the genetic diversities and population structure of six natural populations of *C. nitidissima* from Guangxi (China) based on RAPD and AFLP approaches. They believed that six populations could be classified into two major genetic groups, and also disclosed significant correlations between genetic groups and geographic distances among samples; Wei et al. (2008) analyzed the genetic structure of 13 populations of *C. nitidissima* by ISSR marker; Vijayan et al. (2009) determined the molecular taxonomy of *Camellia* by analyzing nrITS sequences. They proposed that 112 species were divided into 8 major clades and 4 isolates; Liu and Gu (2009) believed that *C. reticulata* and *C. japonica* were closely related and that *C. japonica* had partly contributed to the origin of the polyploidy of *C. reticulata*, based on GISH (genomic *in situ* hybridization).

The previous investigations provided useful information for deeply exploring *Camellia* genetic diversities or relationships. However, challenges still exist due to the lack of enough molecular information to establish a general standard for the classification and identification of *Camellia*. Therefore, more investigations are still needed to further explore the genetic diversities of *Camellia*.

MATERIAL AND METHODS

Plant material and genomic DNA extraction

A total of 15 *Camellia* cultivars were collected from Zhejiang Hongxin Garden Company (Wenzhou, China). They were representative of the major cultivars in Wenzhou, China (Table 1). Genomic DNA was extracted from tender leaf tissue with a modified CTAB method (Porebski et al., 1997). The purity and quantity of genomic DNA were determined by spectrophotometry (Biomate 5, Thermo Electron Corp.) and 1.5% agarose gel electrophoresis (Amersham Ecl, Electrophoresis power supply-Eps.301), respectively.

Table 1. Samples' name, serial number and morphological traits.

Code	Name	Petal color	FD (cm)	BP
N1	Wuhe PZ (*C. reticulata*)	Red	8-10	Mar-Apr
N2	Xiuqiuhua (*C. japonica*)	Red or dark red	7-9	Nov-Mar
N3	Nuo'Er'Si (*C. japonica*)	Red	7-9	Nov-Mar
N4	Manao (*C. reticulata*)	Pink	7-9	Dec-Apr
N5	Dafugui (*C. japonica*)	Pink	8-11	Feb-Mar
N6	Seroyang (*C. japonica*)	Red with white piece	8-11	Feb-Apr
N7	Chidan (*C. japonica*)	Red	10-13	Feb-Mar
N8	Yuanyang FG (*C. japonica*)	Red with white piece	11-13	Feb-Mar
N9	Fenshi YJ (*C. japonica*)	Pink	5-6	Feb-Mar
N10	Baishi YJ (*C. japonica*)	White with pink strip	5-6	Feb-Mar
N11	Qixinghong (*C. japonica*)	Dark red	7-8	Mar-Apr
N12	Huajixiang (*C. japonica*)	Pink with white piece	11-13	Jan-Mar
N13	Mark Allan (not identified)	Wine red	11-13	Sep-Feb
N14	Feibai FR (*C. japonica*)	Red	8-13	Jan-Mar
N15	Mary Agnes Patin (not identified)	Rosy red	11-14	Jan-Feb

Camellia names according to Gao et al. (2005). FD = flower diameter; BP = blooming period.

RAPD-PCR amplification

Fifteen 10-nt random primers (Table 2) were screened out of 202 primers (S1-S50, S100-S150, S200-300), which were synthesized by the Shanghai Bioengineering Company (Shanghai, China). PCR amplification reaction and program were as described by Wang et al. (2010). All PCR products were separated by 1.2% agarose gel electrophoresis in 0.5X TBE buffer (44.5 mM Tris-HCl; 44.5 mM boric acid; 1 mM EDTA). The bands were then visualized by ethidium bromide staining for 15-20 min and photographed under UV light by a Gel Doc-It Imaging system (UVP, Bio Doc Co., USA). The parallel amplification experiments were performed at least two times until distinct and reproducible bands occurred.

Table 2. Primers and their sequences used in this study.

Primer	Sequence	Primer	Sequence	Primer	Sequence
S10	CTGCTGGGAC	S43	GTCGCCGTCA	S256	CTGCGCTGGA
S11	GTAGACCCGT	S112	ACGCGCATGT	S263	GTCCGGAGTG
S20	GGACCCTTAC	S128	GGGATATCGG	S265	GGCGGATAAG
S32	TCGGCGATAG	S216	GGTGAACGCT	S267	CTGGACGTCA
S39	CAAACGTCGG	S254	TGGGTCCCTC	S300	AGCCGTGGAA

Data processing and cluster analysis

All gel bands were scored as present (1) or absent (0) in each genotype for each primer. The band number scoring data were used to calculate the similarity (S) matrixes as described by Nei and Li (1997). A dendrogram was constructed based on similarity matrix using cluster analysis with the unweighted pair group method with arithmetic average (UPGMA).

RESULTS

Analysis of genotype polymorphism among the *Camellia* cultivars

Figure 1 shows the amplification results from partial 10-nt random primers (S20, S112) to 15 genomic DNA samples, and amplification size ranged from 0.3 to 4.0 kb or so. A total of 1935 bands were observed at 88 amplification loci on 15 electrophoretic gels, and 68 amplification loci were polymorphic, indicating percentage of polymorphic loci of 77.3% with an average of 4.5 polymorphic loci per primer. A similarity coefficient between 15 samples ranged from 0.5419 to 0.7933 (Table 3), in which the lowest value was 0.5419 between N4 (Manao, *C. reticulata*) and N14 (Feibai FR, *C. japonica*), implying the farthest genetic relationship, and the largest value was 0.7933 between N7 (Chidan, *C. japonica*) and N8 (Yuanyang FG, *C. japonica*), disclosing the closest genetic relationship among the 15 samples.

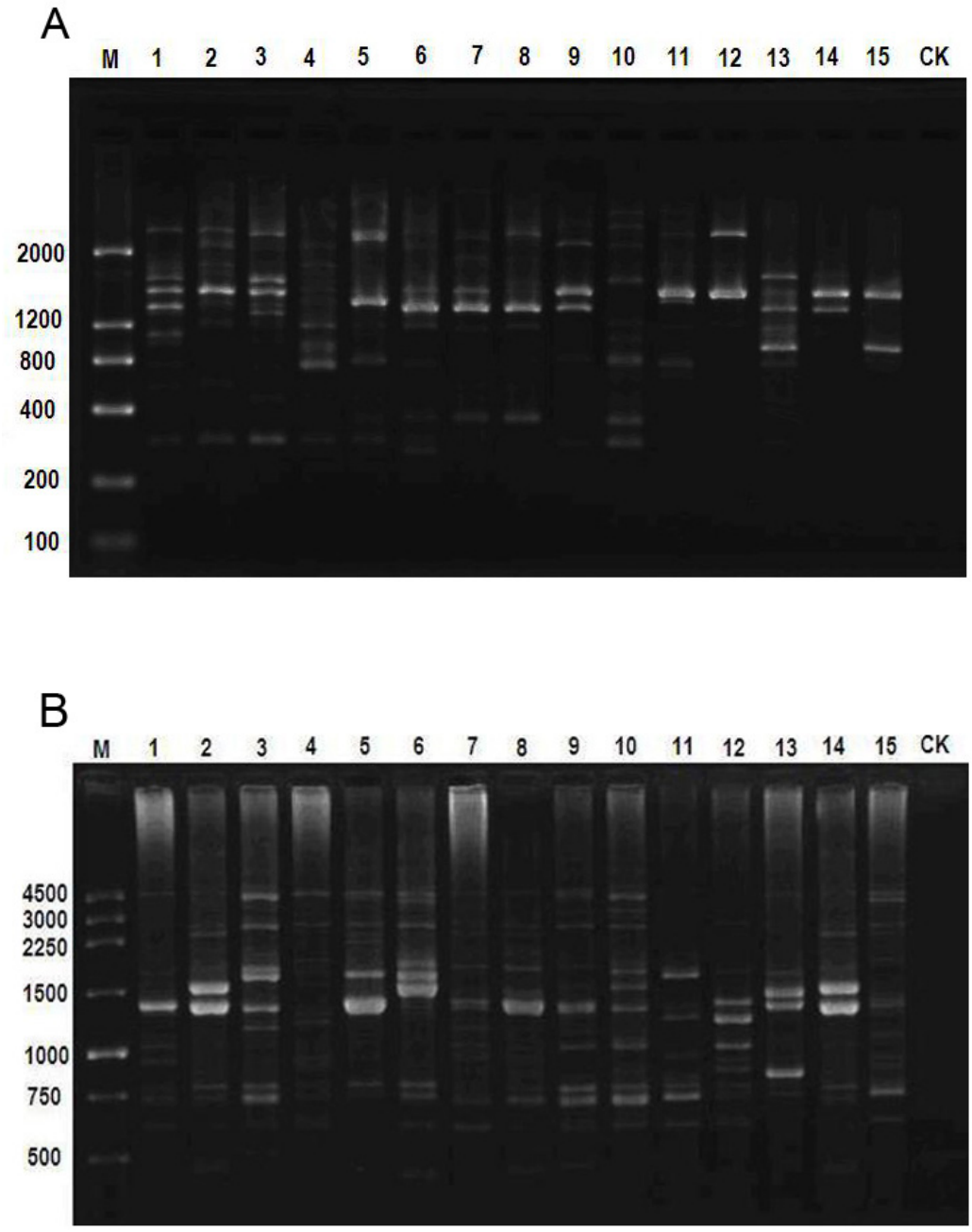

Figure 1. A. Amplification products by polymerase chain reaction using primer S20. **B.** Amplification products by polymerase chain reaction using primer S112. *Lanes 1-15* are identified in Table 1. M = DNA marker; CK = the negative control.

Table 3. Genetic similarities of 15 ornamental *Camellia* cultivars in Wenzhou (China).

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15
N1	1.0														
N2	0.7598	1.0													
N3	0.7151	0.7095	1.0												
N4	0.6872	0.6816	0.7039	1.0											
N5	0.7151	0.6760	0.7430	0.7151	1.0										
N6	0.7095	0.7039	0.7598	0.7318	0.7821	1.0									
N7	0.7430	0.6704	0.7263	0.6760	0.7151	0.7318	1.0								
N8	0.6812	0.6313	0.6536	0.6480	0.6983	0.6927	0.7933	1.0							
N9	0.7318	0.6592	0.6704	0.6983	0.7151	0.7095	0.6536	0.7039	1.0						
N10	0.6816	0.6648	0.6983	0.6480	0.6872	0.7039	0.6592	0.6201	0.6704	1.0					
N11	0.7151	0.6648	0.6425	0.6480	0.6536	0.6704	0.6927	0.6313	0.6927	0.6425	1.0				
N12	0.6648	0.6592	0.5922	0.6425	0.6145	0.5978	0.6872	0.6145	0.6536	0.5922	0.7151	1.0			
N13	0.6480	0.6313	0.5754	0.5587	0.6313	0.6034	0.6257	0.5978	0.6480	0.5754	0.6983	0.6480	1.0		
N14	0.6536	0.6369	0.5810	0.5419	0.6145	0.6089	0.6536	0.6034	0.6201	0.6034	0.6145	0.6648	0.6592	1.0	
N15	0.6809	0.6369	0.6034	0.6089	0.6145	0.6425	0.6536	0.6034	0.6313	0.6034	0.6704	0.7095	0.6592	0.6872	1.0

Cluster analysis

Figure 2 discloses that 15 *Camellia* cultivars were distinctly clustered into two groups with a similarity coefficient cut-off of 0.65: N1-N10 in the first group and N11-N15 in the second. In addition, both groups could be divided at a similarity coefficient of 0.68: nine samples (N1 to N9) were in the same cluster while N10 was isolated from the first group, whereas five samples (N11 to N15) were divided into three parts, in which N11, N12, N15 clustered together while N13, N14 were isolated from the second group.

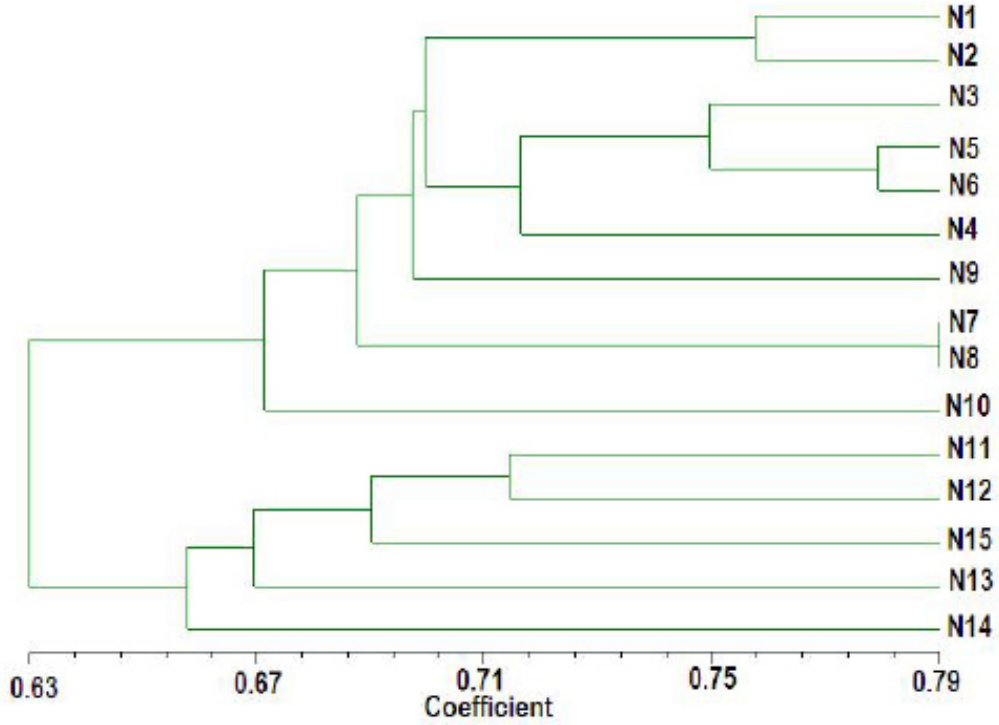

Figure 2. UPGMA dendrogram of 15 samples by RAPD analysis using 15 primers.

Morphological trait analysis

Table 1 describes three important morphological traits of 15 samples: petal color, flower diameter and blooming period. All samples shared approximate petal color (red or pink) with the exception of sample N10 (white). For flower diameter, two samples (N9 and N10) belonged to small-sized flowers (5-6 cm), five samples (N7, N8, N12, N13, N15) to large-sized flowers (10-13 cm), and the remaining 8 samples to middle-sized flowers (7-10 cm). For blooming period, three samples (N2, N3, N4) belonged to early blooming cultivars, and two samples (N1 and N11) to late blooming cultivars, whereas the remaining 10 samples belonged to intermediate blooming cultivars. Figure 3 shows the flower organ and color of some *Camellia* cultivars.

Figure 3. Flower shape and color of partial *Camellia* cultivars (Upper: N9, Fenshi YJ (*C. japonica*) and N4, Manao (*C. reticulata*); Lower: N8, Yuanyang FG (*C. japonica*) and N10, Baishi YJ (*C. japonica*)).

DISCUSSION

Different molecular marker approaches or techniques have their particular sensitivity and applicability. In comparison, the RFLP approach covers a small volume of information, ISSR markers have limited binding sites to genomic DNA, and AFLP markers feature a complicated operation process due to relatively high stability. For SNP analysis, it is costly and labor-intensive, though the approach could disclose relatively true genetic diversities. Recently, new approaches such as nrITS and plastid DNA sequence analysis have been applied to explore genetic diversities, but both approaches also disclose a small volume of local information of the genome. For RAPD markers, there are some doubts because of their relatively low reproduction (or stability). However, this problem of reproduction (or stability) could be easily solved by using high-quality genomic DNA, optimized reaction system and program in a standard operation. In the present study, we acquired clear, stable and reproducible amplification bands with an optimized system and procedure (Wang et al., 2010). Therefore, RAPD markers still provide a rapid and useful technique to investigate genetic diversities at the whole genome level (Ahlawat et al., 2010; Ahmad et al., 2010; Leal et al., 2010).

Camellia features rich genetic resources through long-time hybridization and artificial selection (Chung and Kang, 1996). The present investigation disclosed that all 15 random primers had rich binding sites (1935 bands at 88 amplification loci) in 15 samples of genomic DNA, and also uncovered 77.3% polymorphic loci. There were large differences in the similarity coefficient (0.5419 to 0.7933), which implied extensive genetic variation among 15 *Camellia* cultivars.

In horticulture, the most important morphological trait could be the floral organ for flowering plant, including floral structure (sepals, petals, stamens, and carpels), flower color and blooming period. These traits are strictly spatially and temporally governed and regulated by a series of genes during the course of different developmental phases, which result in the synthesis of flavonoids and carotenoids, pH change of enchylema, development of floral organs, and finally formation of floral structures (Vandenbussche et al., 2003; Sablowski, 2010). Here, three morphological traits, including petal color, flower diameter and blooming period, were selected for study. In the first group, morphological analysis disclosed that nine samples (N1 to N9) featured similar petal colors (red), while sample N10 did white petal (Table 1). Cluster analysis showed that 9 samples (N1 to N9) clustered together, while sample N10 was isolated at a similarity coefficient of 0.68 (Figure 2). It seemed that there was a correlation between cluster analysis based on molecular markers and morphological analysis based on flower color in the first group. Additionally, samples N9 and N10, were derived from the same *Camellia* cultivar - Shiyangjing (Chinese traditional cultivar) and share highly morphological similarities except flower organ, particularly petal color (Table 1; Figure 3). However, they represented relatively low genetic similarity (0.6704) (Table 3), and were divided into different clusters at a similarity coefficient of 0.68 (Figure 2), which implied that gene(s), governing or regulating metabolic pathways of petal color, could play an important role in RAPD analysis (Wang et al., 2010). For sample N8 (Yuanyang FG), a bud variant of sample N7 (Chidan), both shared extremely high morphological similarities in petal color, flower diameter size and shape, and blooming period (Table 1). Theoretically, compared with the other 13 samples, N7 and N8 should share the highest genetic similarity and cluster most closely, and information data (the highest similarity coefficient of 0.7933 between N7 and N8) and cluster results did confirm this presumption (Table 3; Figure 2). Therefore, we believe that RAPD analysis was sensitive enough to distinguish genetic diversities among *Camellia* cultivars.

In the second group, five samples featured similar petal color (red) (Table 1). However, distinct differences were observed when considering flower diameter or blooming period. For N14, its flower diameter ranged from 8 to 13 cm (from medium to large flower), while that of N11 only from 6 to 8 cm (small flower) and those of N12, N13, N15 were similar (11-13 cm; large flower). As for blooming period, five samples also showed distinct differences: N11 (late blooming: March-April), N13 (early blooming: September-February of the following year) and N12/N14/N15 (intermediate time blooming: January-March). Obviously, parallel correlations failed to occur between morphological analysis (flower diameter or blooming period) and cluster analysis based on molecular markers (Figure 2). The failure to detect correlations possibly resulted from the following two phenomena: 1) Most traits are often governed and regulated by a number of genes, whereas each gene possibly governs and regulates more than one trait; 2) Binding sites of each primer often cover the whole genome not only corresponding gene sections, so the whole genome DNA (including all genes) could contribute to RAPD results. That is, all genes governing flower diameter, blooming period, leaf type, growth potential, tree shape, for example, play different roles in RAPD analysis, in which some genes could play dominant roles and others small roles in RAPD analysis. Therefore, extremely complex amplification information interferes with distinctly parallel relationships between genes and traits.

In the past (and even in the present), plant breeders often select hybridization targets with large differences in morphological traits, scarcely considering their genetic background. Therefore, in practice, breeders often have to spend much time in trying luck to get ideal

trait(s) due to the fact that some hybridization combinations feature distinct morphological differences but they possibly share high genetic similarity in their genomes. Therefore, breeders have few chances to obtain ideal traits when performing hybridization. Therefore, it is necessary and urgent for plant breeders to explore plant genetic diversities at the genome level. Luckily, molecular approaches, such as genetic-modified technique or molecular marker-assisted breeding, have been successfully applied in practice (Varshney et al., 2005; Collard and Mackill, 2008). In this study, based on a comprehensive analysis of morphological traits (petal color, flower diameter, blooming period) and genetic diversities, we found that the combination N10/N13 was the most suitable hybridization targets, and the combination N4/N14 was the second.

ACKNOWLEDGMENTS

Research supported by the Department of Education of Zhejiang province, China (Project #Y200803397), Science and Technology Bureau of Wenzhou, Zhejiang, China (Project #N2005A012). The authors wish to thank Hongxin Lin, Dahe Zhang and Xizhi Chen for kindly providing materials.

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