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Genetic relationships in a germplasm collection of *Camellia japonica* and *Camellia oleifera* using SSR analysis

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ABSTRACT. *Camellia japonica* produces different color and bigger flowers, widely being used for gardening green in southern China. However, cultivars were introduced from different regions, but their origin and pedigree information is either not available poorly documented, causing problems in authentication. Many low-yield trees in *Camellia oleifera* forests have been used as stocks for grafting *C. japonica*. However, the survival rate of grafts between these two species is related to genetic relationship between stock of *C. oleifera* and scion of *C. japonica*. We used simple sequence repeat (SSR) markers to genotype 41 *C. japonica* cultivars from different regions, as well as nine genotypes of *C. oleifera* in China. Twenty-one SSR markers generated 438 alleles, with an average of 20.85 alleles per locus. All alleles were used to generate Dice coefficients between two genotypes of all genotypes of these two species. Cluster analysis based on SSR data clustered genotypes showed clustering of genotypes into groups

that agreed well with their taxonomic classification and geographic origin. Cultivar 'Damaonao' was a large tree with flowers of composite color, and showed the most genetic distance from other *C. japonica* cultivars and *C. oleifera* genotypes in the cluster analysis. The cultivars of *C. japonica* are distinct from genotypes of *C. oleifera*. The results for cultivars of *C. japonica* also revealed the presence of different cultivars with the same name, and identical cultivars but with a different name. SSR profiles can improve *C. japonica* germplasm management, and provide potential determine correlations between genetic relationship and graft compatibility among scions of *C. japonica* and genotypes of *C. oleifera*.

Key words: *Camellia japonica*; SSR marker; Genotypes; Genetic relationship; Germplasm management

INTRODUCTION

The genus *Camellia* (Theaceae) includes about 250 species of evergreen shrubs and trees, many of which are notable for their economic and ornamental value. Notably *Camellia sinensis* produces tea, *Camellia oleifera* produces edible oils, and *Camellia japonica* produces ornamental flowers. *C. japonica*, an insect- and bird-pollinated, broad-leaved evergreen tree, is widely distributed in China, Japan and the southern Korean peninsula (Chung et al., 2003). Native species are known for their large, conspicuous flowers (1-12 cm in diameter) with five to nine petals. Flower colors vary from white to pink to red, while yellow flowers are found only in South China and Vietnam. Our observation showed that *C. japonica* reaches sexual maturity and produces flowers after a juvenile period of about 5-10 years. At present, the *C. japonica* is widely being used for gardening green, such as family courtyard, surrounding house, public garden, and urban road.

To improve flower color and size, over 100 cultivars of *C. japonica* were successfully selected and bred in East Asia using conventional breeding programs such as crossing and pedigree selection. In China, over 50 cultivars were selected or introduced from different regions, but their origin and pedigree information is either not available or poorly documented, causing problems in authentication. For example, someone could sell one renamed cultivar in the market, but in fact this cultivar could be introduced from other breeder and then was renamed. In addition, recently, the plants of *C. japonica* with tall tree crown are becoming more and more popular in the market of southern China. However, the plant growth of *C. japonica* is very slow; it is about 10-20 years until one plant with tall tree crown.

The tea oils extracted from the seeds of *C. oleifera* contain bioactive compounds with powerful nutritional and medicinal value (Estevinho et al., 2012; Jin, 2012; Salinero et al., 2012). This seed oils contain large concentrations of oleic acid (Lee et al., 2007) and other bioactive compounds such as vitamin E and squalene (Estevinho et al., 2012). Oleic acid oils lower cholesterol and triglycerides and help prevent cardiovascular diseases (Tholstrup et al., 2004). The triterpenoid saponin improves immune function, enhances antibacterial and antiviral activities, and has antimutation and antioxidation properties (Zhan, 1999). In China, *C. oleifera* has been being widely cultivated for oil and eco-environmental restoration since 1960, reaching a production area of 3.3 million ha in 2015. About 73% of the forests were planted in the 1960's and 1970's using heterogeneous

seedling mixes. Many of these trees have a fresh fruit yield of 0-1.5 kg/year and were considered "low-yield trees". These low-yield trees have strong resistance to drought and poor soil and growing vigor, with 100 + year lifespans; which have been used as stocks for grafting *C. japonica* by the method of high-grafting and change-crown (Zhang et al., 2015). Then, the tall tree crown could be formed 2-3 years after grafts. The survival rate of grafts between these two species is decided by the genetic relationship between stocks of *C. oleifera* and scions of *C. japonica*; however, there is no report on genetic relationship between these two species.

Morphological traits are important indices for taxonomic classification of *C. japonica* cultivars, but they cannot adequately reveal genetic differences because phenotypic traits are highly influenced by environmental conditions (Fufa et al., 2005; Li et al., 2009). In contrast, molecular markers are not influenced by the environment, and can be used to distinguish germplasm (Ali et al., 2008). Several kinds of molecular markers, such as random amplified polymorphic DNA, inter-simple sequence repeat (ISSR), amplified fragment length polymorphism, and sequence-related amplified polymorphism, have been widely used to identify genetic relationship and diversity of germplasm, to construct linkage map and fingerprinting, and to reveal phylogenetic evolution (Wang et al., 2011; Zaloglu et al., 2015; Ding et al., 2016). However, these dominant markers have limited application in marker-assisted breeding and integrating genetic linkage maps, especially in heterozygous out-breeding perennial species in the genera such as *Camellia* (Zaloglu et al., 2015).

Simple sequence repeat (SSR) or microsatellite markers are tandemly repeated DNA sequences that occur randomly and frequently in eukaryotic genomes. Compared to dominant markers, SSR markers are co-dominant, highly reproducible, multi-allelic, and widely used in plant sciences (Hajmansoor et al., 2013) for germplasm characterization (Varshney et al., 2005; Peng et al., 2015; Queiroz et al., 2015), identification of genetic diversity (Gurcan et al., 2015; Jo et al., 2015; Mahjbi et al., 2016; Mornkham et al., 2016; Neiva et al., 2016), germplasm fingerprinting (Zhang et al., 2015), and integration of genetic linkage maps (Lai et al., 2013). For *C. sinensis*, SSR markers have been widely used to identify germplasm genetic diversity (Taniguchi et al., 2014), construction of linkage maps (Tan et al., 2013) and DNA-fingerprinting (Ujihara et al., 2009; Ma et al., 2010). Based on the *C. sinensis* sequences in the NCBI (National Center for Biotechnology Information), three SSR markers for *C. japonica* were developed (Ueno and Tsumura, 2009). Recently, developed 15 genic-SSR markers for *C. oleifera* were developed based on transcriptome sequencing. These successfully amplified PCR products with expected size and polymorphisms in 15 cultivars of *C. japonica* (Jia et al., 2014). Fifty-two SSRs developed from transcriptome sequencing revealed polymorphism in 20 cultivars of *C. oleifera*, with number of alleles per locus ranging from 2 to 15 and expected heterozygosity values from 0.269 to 0.888. Cross-species transferability rates in *Camellia chekangoleosa* and *C. japonica* were 90.4 and 78.8%, respectively (Jia et al., 2015).

In the present study, 21 genic-SSR markers were developed using RNA-Seq. These markers were used to authenticate 41 *C. japonica* cultivars and nine genotypes of *C. oleifera*, and to estimate their genetic relationships. The application of SSRs profiles in pre-breeding, breeding, and identification of *C. japonica* germplasm collections is discussed, as well as a potential strategy for improving grafting survival rates based on genetic coefficients between stocks of *C. oleifera* and scions of *C. japonica*.

MATERIAL AND METHODS

Plant material

Fifty genotypes were used in this study: 41 genotypes of *C. japonica* cultivars (11 from Yunnan Province and 30 from Zhejiang Province). Additional nine genotypes belonged to *C. oleifera* ('Changlin27' from Jiangxi Province, 'Xiang210' and 'Xianglin5' from Hunan Province, and 'NP1', 'NP2', 'NP3', 'NP56', 'NP57', and 'NP58' from Guizhou Province). The cultivars of *C. japonica* were selected because they have been planted widely in southern China, grow well and have many desirable agronomic traits (e.g., different color and size of flowers; Figure 1 and Table 1), 11 of them were selected and bred in Yunnan province and the remaining 30 were selected and bred in Zhejiang province. The genotypes of *C. oleifera* have been widely planted in southern China, which produced many low-yield trees with strong resistance to drought and poor soil and growing vigor. These trees could provide full of stocks for grafts of scions of *C. japonica*.

Figure 1. Several cultivars of *Camellia japonica*: **A.** Beila; **B.** Chalisidun; **C.** Chidan; **D.** Dalicha; **E.** Damanao; **F.** Dasongzi; **G.** Dandinghe; **H.** Hentiangao; **I.** Hualuzhen; **J.** Huaxianzi; **K.** Kuancaidai; **L.** Liujiaodahong; **M.** Moshucheng; **N.** Nantianwushi; **O.** Qiumudan; **P.** Shizitou; **Q.** Tongzimian; **R.** Xinsonghua; **S.** Zhenghuangqi; **T.** Zihudie.

Table 1. Genotypes of *Camellia japonica* and *Camellia oleifera* used in this study.

Species	Origin	Genotypes (No.)	Traits
Camellia japonica	Yunnan Province	Jinxinfurong (1)	Semidouble and lotus type, red, base connate and cylindrical flowers, with 6-7 cm diameter and 5-7 petals
		Dalidiechi (2)	Semidouble and butterfly wing type, red flowers, normal male and female, big and flat outer petals, shaped inner petal
		Xuejiao (3)	Semidouble and white flowers with red outer petals, elliptic to oblong leaves, flower diameter 12-15cm, about 26 petals, florescence: Jan.-Feb.
		Dandinghe (4)	Semidouble, red, slightly cupped flowers, inner petals with pinkish white stripe, silver white haired rest on other petals
		Songzikui (5)	Rose-petal and red flowers, few stamens, pistil development or abortion, flower diameter 6-7 cm, florescence: early Feb. - early March
		Fengshancha (6)	Rose-petal and red flowers, stamens numerous, flower of multicomponent, pistil degradation; flower diameter 10 - 13 cm, florescence: Feb.- March
		Dalicha (7)	Doulbe, peony and purple red flowers, 12-22 cm flower diameter, over 30 petals, florescence: Jan.-March
		Zaodudan (8)	Peony and purple red flowers, flower diameter 10-12 cm
		Shizitou (9)	Peony and red flowers, flat outer and twist inner petals, stamens numerous, multicomponent mixed born petals pistil most tortuous, florescence: Jan.- March
		Dazipao (10)	Peony and deep red flowers; imbricate, rarely in the round rolling slightly undulating irregular; stamens numerous petal, pistil degeneration; flower diameter 11 - 13 centimeters, maximum to 16 cm; florescence: Jan. - March
		Damanao (11)	Double, peony and multi-color flowers, flat outer and twist inner petals, stamens numerous, multicomponent mixed born petals pistil most tortuous, florescence: Jan.- March
	Zhejiang Province	Dasongzi (12)	Semidouble and dark red flowers, petals reflexed, central with a tube of stamens, 8 cm flower diameter, florescence: late Feb. -March
		Wuyepiaoyun (13)	Semidouble to peony type, black red flowers with white cloud shaped patches, petals valgus, with a golden stamen
		Meibuer (14)	Semidouble and white with pink spots flowers
		Jiaomeiren (15)	Semidouble to peony type or even completely double leaf type; pale pink flowers; giant flowers, with an average diameter of 13.5 cm, 5-8 cm thick; opened in early flowering
		Xinsonghua (16)	Semidouble and red flowers, petals 4-5 round center with fine petals and stamens, flowers resembling pine nuts, pine nuts but large petals reflexed, the upper end of the recess; florescence: Feb. - March
		Nantianwushi (17)	Double or peony and rose red with white patches or clouds flower, middle or large flower
		Zhenghuangqi (18)	Semidouble or double type; yellow flower
		Tongzimian (19)	Rose-petal and pink white flower, petals imbricate arrangement, 5-7 wheel, left a few pieces in stamen, pistil became flat
		Manao1 (20)	Rose-petal and pink red flower, 12-15cm flower diameter, about 30 pieces of petals
		Manao2 (21)	Rose-petal and red flower, 12-15cm flower diameter, about 30 pieces of petals
		Huaxianzi (22)	Complete type, rose-petal and red flowers, large flowers, over 30 pieces of petals
		Hentiangao (23)	Rose-petal and deep red flowers, petals imbricate arrangement, 5-7 wheel, left a few pieces in stamen, pistil became flat
		Nongxiang (24)	Peony and pink red flowers, about 30 pieces of petals, about 10 small petals mixed with a few golden stamens
		Shanghainvshi (25)	Peony and pink red flowers, 13-15 cm flower diameter
		Huopubu (26)	Peony and red flowers, corolla dazzlingly beautiful blood red, petals imbricate; stamens numerous, arranged in the 2-6 round, often at the lower part of outer filament synthetic filaments and tube, and petals basally connate
		Moshucheng (27)	Peony type, red flowers, floral color type is constantly changing, fire shape with white patches, best peony cultivar with white medium flowering
		Qiududan (28)	Peony and orange red flowers, 10-14 cm flower diameter, florescence: Late Sep. - Dec., the difference with the flower peony is no white petals on the petals of autumn peony, there is no macular on the leaves, while the flower peony petals have white patches, sometimes with macular leaf
		Hualuzhen (29)	Peony, red with white patches flowers, petals slightly wrinkled, white patches or broad stripes, petals into a few wheel arrangement; golden yellow stamens in the central; medium to large flower, growth vigor, florescence: Feb. - March
		Kuancaidai (30)	Peony type, milk white pink spots with red lace flowers, dark green and thick leaves, 12-15 cm flower diameter, a faint fragrance
		Heimudan (31)	Peony and deep red flowers, 9-11 cm flower diameter, petals from 5 to 7 round; stamens a few scattered petals, florescence: Jan. - March
		Zhushazipao (32)	Dark red flowers is dark purple, 13-15 cm flower diameter, petals 40 to 50 pieces, from outside to inside becomes small and the clip was born in stamens between petals, pistil degradation, florescence: Feb. – March
		Zihudie (33)	Peony and deep red flower, 8-10 cm flower diamter
		Qiaoyi (34)	Complete type, bright red, white and red-white flowers
		Chalisidun (35)	Peony type to rose double type, petals have varying degrees of white patches, 12-15 cm flower diameter
		Liujiaodahong (36)	Obovate and larger leaves, complete type and bright red flower with 7-8 diameter and 70-80 petals forming 6-7 wheels, base free petal, degeneration of male and female, florescence: May-June
		Zhinv (37)	Complete type and pink red flowers, more than 100 petals spiral, 11 cm flower diameter, florescence: Oct.- Feb.
		Meiguodahong (38)	Complete type, deep red flower, 10-20 cm flower diameter, over 100 petals imbricate or arranged in a spiral like arrangement
		Chidan (39)	Completely double type and deep red flowers, over 70 petals arranged in 8-9 round, 10 cm flower diameter, petals inverted tip oval, apex depression, texture thick
		Beila (40)	Complete type, black red flowers, large flower
		Liujiaoyinsi (41)	Complete type and silver-red flowers, 8-10 cm flower diameter
Camellia oleifera	Hunan Province	Xiang210 (42), Xianglin5 (43)	Complete type and white flowers, bigger and cyan-yellow fruits, alternate bearing, strong resistance to drought, cold and west, well growth vigor
	Guizhou Province	NP1 (44), NP2 (45), NP3 (46), NP56 (47), NP57 (48), NP58 (49)	Complete type and white flowers, small and cyan fruits, low-yield trees, well growth vigor, full of resources as stocks of grafting *Camellia japonica*
	Zhejiang Province	Changlin27 (5)	Complete type and white flowers, middle and red fruits, alternate bearing, well growth vigor

Young and fully expanded leaves were collected in these provinces in May 2015 and immediately stored in plastic bags with silica gel at room temperature, until DNA extraction about one month after young collection. Only one tree per genotype was selected for collecting leaves, because the different trees are from the same genotypes by vegetative propagation. The geographic and breeding origins of all genotypes were described in Table 1.

DNA extraction and SSR-PCR amplification

Genomic DNA was extracted from 100 mg of dry young leaves using a modified CTAB method following the procedure described by Ruan et al. (2004). DNA quality and quantification were checked by Nanodrop 2000c (Thermo scientific, Willington, CT, USA) and visualized on a 1% agarose gel. The Nanodrop 2000 conditions and settings are that 1.5 μL gDNA solutions were dropped into the detector on the condition of gDNA type of nucleic acids module. Agarose gel running conditions are 1% (w/v) agarose gels electrophoresis using the 0.5X TE buffer (44.5 mM Tris, 44.5 mM boronic acid, 1 mM EDTA, pH 8.3) on the conditions of 200 V for 30 min and visualized in a GelDoc-It2 Imaging Systems after staining with Gold View I. The genomic DNA was diluted to 20 ng/μL using Tris-EDTA buffer, and stored at -20°C in a refrigerator with for SSR analysis.

Based on the data of RNA-seq of the leaf of *C. japonica*, a total of 34,833 SSRs distributed in 27,552 unigenes were identified as potential molecular markers. The primers of 138 SSR markers (Sangon Biotech, Shanghai, China) were designed by the Primer5 software based on the complementary sequence of both ends of SSR. Optimal annealing temperature per primer combination was based on the number and quality of polymorphic fragments at 56°, 56.3°, 56.8°, 57.6°, 58.6°, 59.4°, 59.8°, and 60°C. Finally, based on amplification and reproducibility in all genotypes, a total of genic-21 SSR markers were selected for identification of genetic relationships between genotypes used in this study.

A 20-μL reaction volume was used for polymerase chain reaction, which contained 2 μL 10X PCR buffer (with 1.6 mM Mg^{2+}, TaKaRa, Dalian, China), 0.4 nmol dNTP (Sangon Biotech, Shanghai, China), 0.5 U Taq polymerase (Takara, Dalian, China), 13.4 μL ultrapure water, 10 pmol each forward and reverse primer, and 40 ng gDNA template. Amplification was performed in a thermocycler (Bio-Rad C1000 Touch TM) with a 4-min initial denaturation at 94°C, followed by 35 cycles at 94°C for 30 s, annealing at 58°C for 30 s, and an extension at 72°C for 30 s. A final extension was performed at 72°C for 10 min and then the product was stored at 4°C. The reactions were then placed at -20°C until gel analysis. The PCR products were loaded onto 8% polyacrylamide non-denaturing gels, and separated by electrophoresis at 150 V for 9 h using a Hoefer SE600 Ruby Standard Vertical Electrophoresis Unit (Amersham Biosciences, Piscataway, NJ, USA) (Lin et al., 2015) and detected by silver nitrate staining. A 1-kb size marker was used as a DNA ladder (Sangon Biotech, Shanghai, China) to estimate the SSR allele band size (Chen et al., 2015).

Data analysis

Gels were scored in a binary format, according to whether a band was present (1) or not (0) by the methods of Chen et al. (2015) and Abdessemed et al. (2015). The genetic diversity of a population was evaluated by the average number of the alleles shared by the individuals in each population and the percentage of polymorphisms (following Lai et al.,

2015). The polymorphism information content (*PIC*) was calculated by the formula $PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$, where p_i and p_j are the frequencies of the *i*th and *j*th allele, and *n* is the number of alleles (Li et al., 2015). The relative frequency of the *j*th allele for the *i*th locus is added across all the alleles for the locus over all genotypes. The Dice coefficient (DC) between pairs of genotypes was measured by the DICE similarity coefficient (Dice, 1945) based on the SSR data using the SIMQUAL module of NTSYSpc ver. 2.02 (Rohlf, 2000).

POPGENE 1.32 was used to evaluate genetic diversity of the different groups, by calculating seven different genetic indices: the number of alleles (N_A), the effective number of alleles (N_E), Nei's gene diversity (*H*), Shannon's information index (*I*), the number of polymorphic alleles (N_p), the percentage of polymorphic loci (*P*).

RESULTS

SSR analysis

Twenty-one SSR markers used generated 438 alleles, 436 (99.5%) of which were polymorphic. The number of alleles per SSR marker ranged from 8 for CJ1030 and CJ1066 to 30 for CJ1100, with an average of 20.85 alleles per locus (Table 2). The PIC values per SSR marker ranged from 0.1800 for CJ2020 to 0.4336 for CJ2051, with an average of 0.3406 per locus. Representative polyacrylamide gel electrophoresis profiles of PCR products amplified using the SSR marker of CJ1066 was shown in Figure 2.

Table 2. Locus and numbers of SSR markers generated for all genotypes.

Locus	Forward (F) and reverse (R) primers	AT (°C)	NB	NPB	Polymorphism	*PIC*
CJ1030	F: AATGCAGTCAAACGAAAGGG R: TGCTGGAGGAATGGATTTTC	58	30	30	100%	0.3737
CJ1031	F: GGGAACAAATTGATGCGATTA R: AATGGTGGTGTTGTTGGGTT	58	21	21	100%	0.3783
CJ1056	F: AATGGCGTCTCCAATTTGTC R: CGGAAGAAGAGACGGTGAAG	58	26	26	100%	0.3616
CJ1057	F: AACCACACACACAACAATCACTC R: TTCACCCCAGAACTGGAAAC	58	28	28	100%	0.2840
CJ1066	F: GGATTTCAGATGCAGACCTTG R: TTTGGGAAAACTAAAGCGGA	58	30	29	97%	0.3012
CJ1071	F: ACAATTGGAGGATGAGTGCC R: AGAGAGTTCCTCCCTCCGAC	58	21	20	95%	0.3876
CJ1078	F: GCAGCGACAGTTCTGTTGAA R: CACCACATCCCCTTCCTCTA	58	23	23	100%	0.2617
CJ1100	F: AAATGGGAAAGATTGGGGAG R: GCCTCTTTCTTCAATTCCCTG	58	8	8	100%	0.3488
CJ2018	F: CCGAGCTCTCAATATCAGGC R: TTCAGGGAAAGGGAAGGAGT	58	22	22	100%	0.1857
CJ2020	F: CACCACGAAGTGATCGTGTC R: ACAAATCGGTCGTGAAGGTC	58	19	19	100%	0.1800
CJ2021	F: GTCATCGAGTGGTGGTGATG R: CACAGGGACAGTGTCGAGAA	58	15	15	100%	0.3263
CJ2024	F: TGGCTACCTTCTTCGTTGCT R: CCATTCTCGACGAATCCAGT	58	15	15	100%	0.3860
CJ2031	F: ACTGTCGGACAAAGCGAGTT R: GGTCTGACGAACCCATTCAC	58	21	21	100%	0.4238
CJ2033	F: GAAATGGCGATTATGGCAAC R: CCCAACGAAACGATAACAGG	58	18	18	100%	0.4127
CJ2036	F: GCTCGTAGGTGTGTTGGGTT R: CAGCCACTTTACCTCCCAAA	58	12	12	100%	0.3119
CJ2049	F: CACAAGGAGGGGGTACGTTA R: CACCTTCCACAGAGGGAGAC	58	28	28	100%	0.2161
CJ2050	F: CTAAGCTTCCCATCAAACGC R: CAGAGGAACAGTCGAGGAGG	58	13	13	100%	0.4019
CJ2051	F: CGTGCCTGCATTGCTAATAA R: GTACCAGTAACTGCGGGGAA	58	18	18	100%	0.4336
CJ2060	F: CGTAACAGCAAGCGACTGAG R: GTGACGAACCGAAACGTACA	58	27	27	100%	0.4096
CJ2062	F: TGCCATAAGACGAACCAGAA R: CCTACTCTCCGCCAGTCTCC	58	15	15	100%	0.3730
CJ2066	F: CACACACACACAAACGCAAA R: GGTGATGATAGGAAAATGATTGA	58	28	28	100%	0.3955

AT: annealing temperature; NB: the number of total bands; NPB: the number of polymorphic bands; *PIC*: percentage of polymorphic loci.

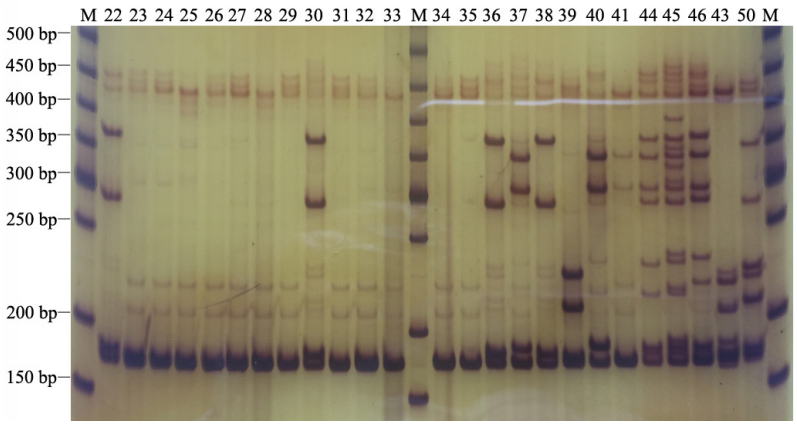

Figure 2. Representative polyacrylamide gel electrophoresis profiles of PCR products amplified using the SSR marker of CJ1066. Number of 22-41 indicated different genotypes of *Camellia japonica* and 43-46 and 50 for genotypes of *Camellia oleifera* in Table 1.

Genetic diversity of *Camellia* cultivars

DCs between each pair of 41 *C. japonica* cultivars ranged from 0.17 to 0.98, with a mean of 0.41 ± 0.13. DCs between all pairs of 50 genotypes ranged from 0.13 to 0.98, with a mean of 0.38 ± 0.13.

Genetic diversities within groups (except Group IV in Figure 3) were assessed using seven genetic parameters (Table 3). For *C. japonica*, the group II cultivars from Zhejiang Province had higher genetic diversity than the group I cultivars from Yunnan Province (Table 3). Group III consisted of nine genotypes of *C. oleifera* showed higher genetic diversity than group I of 10 genotypes and group II of 30 genotypes of *C. japonica* (Table 3). For example, N_E of group III was 1.32, and was 1.25 and 1.18 for groups II and I, respectively. *H* of group III was 0.21, and was 0.17 and 0.11 for groups II and I, respectively. *N*p of group III was 272, and was 243 and 71 for groups II and I. *PIC* of group III was 0.40, and was 0.34 and 0.33 for groups II and I, respectively.

Cluster analysis

At a DC of 0.351, 50 genotypes were clustered into three major groups (I, II and III) except for cultivar 'Damanao' from Yunnan Province (IV) (Figure 2). The cluster analysis showed groupings of genotypes, which agreed with their taxonomic classification and geographic origin. Cultivar 'Damaonao' was a large tree with flowers of composite color, and showed the most genetic distance from other *C. japonica* cultivars and *C. oleifera* genotypes in the cluster analysis. In contrast, all other genotypes had single-colored flowers, and most of them were shrubs or small trees.

Group I included 10 *C. japonica* cultivars from Yunnan Province. At a DC of 0.648, Group I was divided into three subgroups: Group Ia consisted of four cultivars 'Jinxinfurong', 'Dalidiechi', 'Dalicha', and 'Xuejiao', which had semi-double and peony type flowers. Group Ib included five cultivars 'Songzikui', 'Shizitou', 'Dandinghe', 'Fengshancha', and 'Dazipao', which had red, purple red or deep red flowers. The remaining 'Zaodudan' cultivar had peony-type red flowers and was in Group Ic.

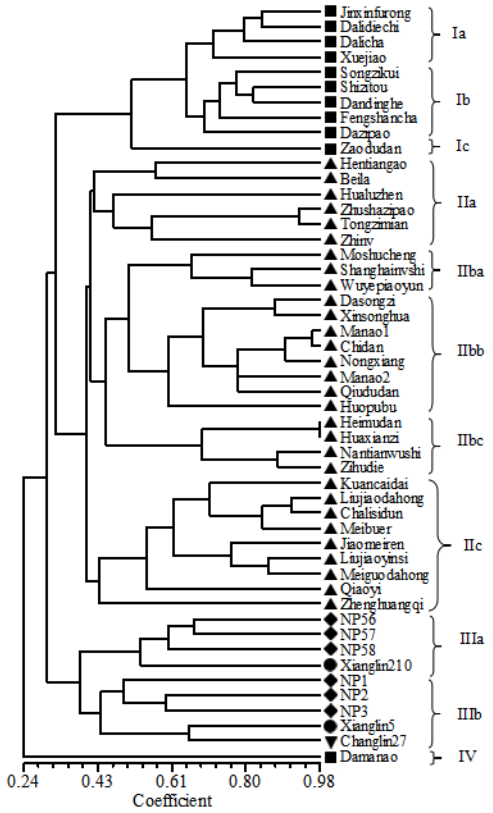

Figure 3. Dendrogram of 50 genotypes revealed by cluster analysis of genetic similarity estimates generated by Dice coefficient based on 438 alleles from 21 SSR markers. Square = geographic location is Yunnan Province; up arrowhead = geographic location is Zhejiang Province; diamond = geographic location is Guizhou Province; circle = geographic location is Hunan Province; down arrowhead = geographic location is Zhejjiang Province.

Table 3. Genetic diversity of *Camellia japonica* and *Camellia oleifera* groups.

Group	*N*	N_a	N_e	*H*	*I*	N_p	*P*	*PIC*
I[i]	10	1.4018	1.1873	0.1148	0.1786	71	40.18%	0.3345
II[j]	30	1.7443	1.2514	0.1651	0.2689	243	74.43%	0.3361
III[k]	9	1.7877	1.3246	0.2089	0.3324	272	78.77%	0.4014

N: the number of genotypes; N_A: observed number of alleles; N_E: effective number of alleles; *H*: Nei's gene diversity; *I*: Shannon's information index; N_p: the number of polymorphic alleles; *P*: percentage of polymorphic loci; *PIC*: polymorphism information content; [i]group I consisted of 10 cultivars of *C. japonica* from Yunnan Province; [j]group II consisted of 30 cultivars of *C. japonica* from Zhejiang Province; [k]group III consisted of nine genotypes of *C. oleifera*.

Group II consisted of 30 *C. japonica* cultivars from Zhejiang Province and shared an identical genetic background. This group was divided into three subgroups at a level of DC of 0.39. Group IIa included six cultivars 'Hentiangao', 'Beila', 'Hualuzhen', 'Zhushazipao', 'Tongzimian', and 'Zhinv'. 'Tongzimian' and 'Zhushazipao' are both shrubs or small trees and were clustered together. 'Huanluzhen' has red flower with white patches, and is different to other three cultivars with single-color flower ('Zhushazipao', 'Tongziman' and 'Zhinv').

Group IIb consisted of 15 cultivars, which is further divided into three sub-subgroups based on a DC of 0.57. The cultivars of the sub-subgroup IIba have similar flower color, but different flower types (semidouble type for 'Wuyepiaoyun' and peony type for 'Moshucheng' and 'Shanghainvshi'). In the sub-subgroup IIbb, the two cultivars of 'Dasongzi' and 'Xinsonghua' with semi-double and red flowers were clustered together; the five cultivars of 'Manao1', 'Chidan', 'Nongxiang', 'Manao2', and 'Qiududan' clustered together and had similar flower color but different floral types (rose-petal type for 'Manao1' and 'Manao2', complete type for 'Chidan' and peony type for 'Nongxiang' and 'Qiududan'). 'Huopubu', which is a shrub or tree, showed the most divergent genetic distance in this sub-subgroup. In the sub-subgroup IIbc, two cultivars of 'Heimudan' and 'Huaxianzi' clustered together had similar flower color, but their floral types differed, e.g., rose type for 'Huaxianzi' and peony type for 'Heimudan'; the two cultivars of 'Nantianwushi' and 'Zihudie' had similar flower color and floral type, and clustered together.

Group IIc consisted of nine cultivars, the 'Zhenghuangqi' showed the most divergent genetic distance in this subgroup and had yellow flowers, which differed from the flower colors of the remaining eight cultivars. The 'Qiaoyi' cultivar had different colored flowers, such as pure red, pure pink red, pure white, and a composite of red and white. The three cultivars of 'Jiaomeiren', 'Liujiaoyinsi' and 'Meiguodahong' with similar flower color clustered together. The two cultivars of 'Liujiaodahong' and 'Chalisidun' had similar floral type and color, and were clustered into one branch in this subgroup; the two cultivars 'Meibuer' and 'Kuancaidai' had white flowers with red-trimmed lace, and also clustered into this branch based on the DC of 0.68.

At a DC of 0.39, group III with nine *C. oleifera* genotypes was divided into two subgroups: Group IIIa consisted of four genotypes, three of which were from Guizhou Province, and the remaining cultivar 'Xianglin210' was from Hunan Province. Group IIIb consisted of five genotypes, three of which 'NP1', 'NP2', 'NP3' from Guizhou Province were clustered into a branch, and the remaining two cultivars of 'Xianglin5' and 'Changlin27' from Hunan and Jiangxi provinces were clustered into another branch.

DISCUSSION

Results revealed for the first time the genetic relationship of genotypes between *C. japonica* and *C. oleifera*, which provides potential determine correlations between genetic relationship and graft compatibility among scions of *C. japonica* and *C. oleifera*. In addition, the SSR profiles in this study clearly identified different genotypes of *C. japonica* in southern China, and showed potential to authenticate cultivars, which are widely used for gardening green. This could improve *C. japonica* germplasm management. There are three reports on SSR markers for *C. japonica*, but 1) only three SSR markers for *C. japonica* were developed and used for genotyping 22 individuals from a Japanese population (Ueno and Tsumura, 2009); 2) Jia et al. (2014) only reported the development of 15 genic-SSR markers for cultivar 'Huashou' of *C. oleifera*, and its ability to successfully amplify PCR products with expected size and polymorphisms in 15 cultivars of *C. japonica* and 18 cultivars of *C. oleifera*, there is no report on genetic relationships among different cultivars, and there cultivars are different from the genotypes in this study; and 3) Jia et al. (2015) only reported cross-species transferability rates of 52-genic SSR markers in *C. chekangoleosa* and *C. japonica* based on SSR markers, which was developed from 20 cultivars of *C. oleifera*. These SSR markers and cultivars of *C. oleifera* are also different from the genotypes in this study, and there is also no report on genetic relationship between cultivars of *C. oleifera* and *C. japonica*.

In this study, the genotypes are from two species of *Camellia*. The SSR profiles of the genotypes showed a high polymorphism for *Camellia* cultivars and for all genotypes. The N_A per SSR marker in this study is higher than the N_A ranging from 2 to 13 in the report of Ueno and Tsumura (2009), in which three SSR markers were used to genotype 22 individuals within a *C. japonica* population. High mean bands per SSR primer combination in this study could be generated from materials with high genetic or geographic differences. In our study, we repeated three amplifications and obtained identical results for primer combinations that generated a high number of alleles (e.g., CJ1030 and CJ1066).

Knowledge of genetic relationships is a key factor for managing genetic resources in a successful breeding program (Amar et al., 2011). Microsatellite genotyping can allow unambiguous identification of germplasm (Dominguez-Garcia et al., 2012; Muzzalupo et al., 2014; Mousavi et al., 2014). Because of their high discriminatory power and typically straight forward interpretation (Taniguchi et al., 2014; Jia et al., 2015), SSR markers have been becoming the preferred choice for genetic identification of *Camellia* germplasm. Couselo et al. (2010) used 14 SSR markers to differentiate two groups of ancient specimens of *C. japonica* grown in gardens of Galicia and Northern Portugal.

In this study, the results of cluster analysis based on SSR profiles are in accordance with their genetic and geographic origin. Twenty-one SSR markers clearly distinguish 39 *C. japonica* cultivars (except for 'Heimudan' and 'Huaxianzi') and nine genotypes of *C. oleifera*. The *C. japonica* cultivars from Yunnan and Zhejiang provinces were clustered into different groups, respectively, in agreement with their geographic origin in the cluster analysis. The genotypes of *C. oleifera* from three provinces (Guizhou, Hunan and Jiangxi provinces) were clustered into one group, indicating genetic accordance. Although the studied cultivars of *C. japonica* and genotypes of *C. oleifera* are from the limited geographic ranges, there is no report on genetic relationships among them using SSR markers in previous studies. The cultivars of *C. japonica* in this study have been planted widely in southern China, grow well and have many desirable agronomic traits, such as bigger and different color flowers (Table 1); the genotypes of *C. oleifera* have been widely planted in Guizhou Province, which provided a plenty of low-yield trees as stocks for grafts of scions of *C. japonica*. Of course, a future study with a greater number of samples from a broader geographic range, and including any wild cultivars would be quite interesting.

In our analysis, the 'Damanao' cultivar clusters into a single group beside both the *C. japonica* group and the *C. oleifera* group, indicating this cultivar may be selected from other subgenus of the *Camellia* genus. In Group III, two *C. oleifera* cultivars 'Xianglin210' and 'Xianglin5' are from the same Hunan province, but the former clusters into one subgroup with three genotypes from Guizhou Province, and the latter appears in subgroup including 'Changlin27' from Jiangxi Province. These indicate the 'Xianglin210' cultivar may be selected from the materials growing in region of Hunan Province nearby Guizhou Province, and the 'Xianglin5' was selected from materials nearby Jiangxi Province.

Molecular markers such as SSR and ISSR can be useful tools to identify and authenticate cultivars (Li et al., 2009; Couselo et al., 2010). Our data revealed that there are two main problems with nomenclature in the *C. japonica* germplasm we studied: 1) some cultivars are the same but have different names, and 2) some cultivars have the same name but are genetically distinct. On the one hand, the DC of 0.98 between 'Heimudan' and 'Huaxianzi' suggests they are likely to be the same cultivar, combining with their similar morphological traits; but they were given different names when selected and bred in China. There was also no

obvious distinction between 'Manao1' and 'Chidan' (DC = 0.96), between 'Zhushazipao' and 'Tongzimian' (DC = 0.93), and between 'Liujiaodahong' and 'Chalisidun' (DC = 0.91); these paired cultivars originate from Zhejiang Province and share a common genetic background and morphological traits. This nomenclature may have resulted from unknown pedigrees and mis-registration of exchange materials with each other, or could have arisen from intentional false naming for commercial gain. On the other hand, three cultivars of 'Manao1', 'Manao2' and 'Damanao' have the same name of 'Manao', but they clustered into different groups, II and IV, respectively (DC between 'Manao1' and 'Damanao' = 0.25, and 0.20 between 'Manao2' and 'Damanao'). Cultivar 'Damanao' was selected in Yunnan Province, and the two cultivars of 'Manao1' and 'Manao2' are bred in Zhejiang Province. Resolving these inconsistencies in genetic identification will help to properly collect and conserve *Camellia* germplasm, and allow a true-to-type verification of material on the commercial market.

The tall tree crown *C. japonica* has a growth period of only 10-20 years, the low-yield but long-lived trees of *C. oleifera* have been used as stocks for grafting scions of *C. japonica* with different size and color of flowers (Zhang et al., 2015). This graft not only could gain tall crown and beautiful *C. japonica* flowers at 2-3 years after grafts, but also could efficiency utilize the low-yield trees of *C. oleifera*. However, grafting compatibility between stocks and scions are important for grafting success (Yan et al., 2012), which is related to their genetic relationships (Belaj et al., 2007).

Cultivated and wild olives show very good grafting affinity.

In this study, the SSR profiles were first used to identify genetic relationships between genotypes of *C. japonica* and *C. oleifera*, and provide a potential strategy for improving grafting success between stocks of *C. oleifera* and scions of *C. japonica*. Based on their genetic relationships, different grafting combinations could be recommended, such as scions of 'Meibuer' with stocks of NP56 (DC = 0.48) (Figure 1C), scions of 'Huopubu' with stocks of 'NP1' (DC = 0.46) (Figure 1D), scions of 'Chalisidun' with stocks of 'NP56' (DC = 0.45) (Figure 1E), scions of 'Liujiaodahong' with stocks of 'NP56' (DC = 0.44) (Figure 1F), scions of 'Shizitou' with stocks of 'NP56' (DC = 0.43), scions of 'Kuancaidai' with stocks of 'NP56' (DC = 0.42), scions of 'Zhenghuangqi' with stocks of 'NP1' (DC = 0.42), scions of 'Heimudan' with stocks of 'NP1' (DC = 0.41) and scions of 'Huaxianzi' with stocks of 'NP1' (DC = 0.41). For example, the survival rate of graft between scions of 'Meibuer' with stocks of 'NP56' was about 84.3% (DC = 0.48). And, it is only about 46.8% between scions of 'Hengtiangao' with stocks of 'Xianglin5' (DC = 0.23), most of scions and stocks will be dead at 4 years after grafting (Ruan CJ, Liu SH, Du W and Mopper S, unpublished data).

Conflicts of interest

The authors declare no conflict of interest.

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