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Full Length Research Paper

Fluorescent amplified fragment length polymorphism (AFLP) analysis of genetic diversity and relationship of Chinese *Rosa rugosa* germplasm resources

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***Rosa rugosa* is an excellent ornamental plant with important ecological, economical and medicinal values in China. Polymorphism amplifications of the genomic DNA of 5 wild *R. rugosa* accessions and 25 cultivars that originated from China with fluorescent amplified fragment length polymorphism molecular markers were investigated in this study to evaluate their genetic diversity and relationships. A total of 1771 clearly identifiable bands (60 ~ 500 bp), including 1704 polymorphic bands, were obtained through amplification using 10 pairs of primers. The average percentage of polymorphic locus was 96.2%, which indicated the high genetic diversity of Chinese *R. rugosa* germplasm resources. Different quantities of specific bands were detected by 10 pairs of primers from 24 accessions, which could be used to identify the specific *R. rugosa* accession. There was high similarity between Chinese *R. rugosa* germplasm resources, as indicated by the similarity coefficient of 30 *R. rugosa* accessions between 0.4977 and 0.8410, with an average of 0.624. Clustering results suggested that wild accessions from different areas of China were remotely related with the ample genetic diversity. Wild accessions and cultivars also had remote genetic relationship. However, the main Chinese *R. rugosa* cultivars had high similarity, low genetic differences and a narrow genetic background.**

Key words: *Rosa rugosa*, genetic diversity and relationship, amplified fragment length polymorphism.

INTRODUCTION

Rosa rugosa is a deciduous shrub of the genus *Rosa*, having prickly stems, pinnately compound leaves and variously colored, often fragrant flowers. The native range of *R. rugosa* includes north-east China (Fu, 1992), northern Japan (Ohwi, 1965), the Korean Peninsula (Ohwi, 1965) and the Russian Far East (Sokolov et al., 1980). In China, wild *R. rugosa* is naturally distributed on the coast and islands of southern Liaoning province, eastern Shandong province and Tumen River estuary Jilin province, and is classified as an endangered species (Fu, 1992).

R. rugosa economic importance is mainly in their petals which are sources of rose oil, especially for the flavor and fragrance industry. In many European countries, it is also used in the production of herbal medicine or foodstuffs rich in ascorbic acid. Furthermore, it is extensively used in the breeding of the cultivated roses, due to its hardiness and disease resistant foliage; it has been widely used as rootstock for grafted roses (Bruun, 2005).

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Abbreviations: AFLP, Amplified fragment length polymorphism; **PPB,** percentage of polymorphic bands; **UPGMA,** unweighted pair group method of arithmetic average.

In China, *R. rugosa* had a long history of cultivation for perfume (over 1300 years), and lots of varieties and cultivars were developed mainly using vegetative propagation (e.g. sport mutation) and seedling selection. Recently, some cultivars were named based on the morphological traits, and correspondingly morphological classification criteria were suggested in our laboratory (Yang et al., 2003; Yu et al., 2005). The morphological characteristics of wild *R. rugosa* of four natural populations in China located at Rongcheng, Muping, Zhuanghe and Hunchun were also investigated. We found that they were very close, and there were no significant morphological variation among the different wild populations (Feng et al., 2009).

Up to now, the unique putative classification system of *R. rugosa* has not been constructed, which has resulted in homonyms and synonyms. In addition, the genomic information of *R. rugosa* and the degree of genetic diversity in highly diversified cultivars was rather poorly known. Therefore, it was very important to use a sensitive and credible molecular technique to detect the DNA variation and identify the specific germplasms of *R. rugosa* in China. Random amplified polymorphic DNA markers had been used to determine the genetic diversity of the four remaining large natural populations of wild *R. rugosa* in China; high genetic variations were detected in *R. rugosa* populations, and most genetic diversity occurred within the populations (Yang et al., 2009). However, genetic diversity among the Chinese *R. rugosa* germplasm resources, especially between wild accessions and cultivars at the molecular level remains unclear.

The amplified fragment length polymorphism (AFLP) (Vos et al., 1995) is a DNA fingerprinting technique that approaches the ideal as a marker system for resolving genetic diversity among individuals, populations and species (Muller and Wolfenbarger, 1999). This technique is highly reproducible and polymorphic, and can be used to survey the overall genetic differences in the nuclear genome in a single assay without any prior sequence knowledge (Vos et al., 1995; Jones et al., 1997). Recently, AFLP technique were widely applied to investigate the genetic relationship among species, closely related cultivars and even clones of plants (Carr et al., 2003; Lanteri et al., 2004; Subudhi et al., 2005; Yildirim and Akkaya, 2006; Mizianty et al., 2006; Yoon et al., 2007; Li et al., 2008; Karimi et al., 2009). As a result of their genome-wide sampling, AFLPs are the markers of choice to reconstruct (species) relationships in evo- lutionary complex groups, such as *Rosa* (Rosaceae) (Wim et al., 2008).

The major goal of this investigation was to assess the genetic diversity in 30 Chinese *R. rugosa* accessions using AFLP analysis, which could provide useful information to address breeding programs, and provide knowledge on the origin and evolutionary history of Chinese *R. rugosa* germplasm resources.

MATERIALS AND METHODS

Plant material

A total of 30 *R. rugosa* accessions were used in this study including 5 wild accessions and 25 cultivars. They were cultivated in the *R. rugosa* germplasm repository of Shandong Agricultural University, and the code, Chinese name, origin and main morphological characteristics of each accession are listed in Table 1.

DNA extraction

Young leaves were collected from each accession and were dried by silica gel immediately. The total genomic DNA was extracted using the plant genomic DNA extraction kit (DP305-02, Beijing Dingguo Biotechnology Co. Ltd., China) according to the manufacturer's directions. DNA concentration was measured by fluorometry (DU 7500, Beckman) and was adjusted to 100 ng/ml. The DNA extractions were stored at -20°C until required.

AFLP analysis

AFLP analysis was performed using *EcoR*I/*Mse*I type AFLP kit (Beijing Dingguo Biotechnology Co. Ltd., China). The nucleic acid sequences of DNA adaptors and primers used in AFLP analysis are shown in Table 2. The primer *MseI* was the FAM fluorescent marker. The procedure was conducted following the instructions of the manufacturer. 2 µl of selective-amplification product was separated by electrophoresis on a 4% denaturing polyacrylamide gel at 50 W (maximum of 3,000 V) for 2.4 h in ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The electrophoresis pattern was collected automatically.

Data analysis

After the electrophoresis, band patterns were done with Genescan 3.1 software (Applied Biosystems, Foster city, CA, USA) and the binary data matrix was constructed. Identification percentage = number of identified cultivars / number of total cultivars × 100%, and the percentage of polymorphic bands (PPB) = (total number of bands - number of common bands) / number of total bands × 100% were calculated. The Dice similarity coefficient matrix was obtained using NTSYS.pc2.11f software (Applied Biosystems, Setauket, NY, USA), and cluster analysis based on the similarity coefficient matrix was performed with unweighted pair group method of arithmetic average (UPGMA).

RESULTS

Polymorphism of amplification products using different primer combinations

AFLP amplification was conducted on the genomic DNA of 30 *R. rugosa* accessions using 10 pairs of primers with good polymorphism and clear bands of amplification products which were chosen from 64 pairs of primers to obtain good amplification results (Figure 1). A total of 1771 clearly identifiable bands (60 ~ 500 bp) was obtained, including 1704 polymorphic bands.

Table 1. Code, name, origin and main characters of 30 Chinese *R. rugosa* accessions in this study.

Code	Chinese name	Origin	Main characters
C1□	Danbanbai	Beijing	Ravidous main branches with a large quantity of punctures, white flowers with single-whorled petals.
C2□	Baojialiyabai	Pingyin City, Shandong Province	Tall and erect plant with ravidous main branches and small quantity of punctures, white flowers with five-whorled petals. Its young shoots are green.
C3□	Sulianxiangshui	Pingyin City, Shandong Province	Erect plant with ravidous main branches with small quantity of punctures, violet red flowers with triple-whorled petals. Its young shoots are green.
C4▲	Mupingyesheng	Muping City, Shandong Province	Low plants with creeping rhizomes, gray main branch with a tight cluster of punctures, pink flowers with single-whorled petals.
C5▲	Rongchengyesheng	Rongcheng City, Shandong Province	Low plant with creeping rhizomes, gray main branches with a tight cluster of punctures, deep pink flowers with single-whorled petals.
C6▲	Zhuangheyesheng	Zhuanghe City, Liaoning Province	Low plant with creeping rhizomes, gray or ravidous branches with a tight cluster of punctures, violet red or deep pink flowers with single-whorled petals.
C7▲	Hunhunyesheng□	Hunchun City, Jilin Province	Low plant with creeping rhizomes, gray branches with a tight cluster of punctures, deep pink flowers with single-whorled petals.
C8▲	HunhunyeshengII	Hunchun City, Jilin Province	Low plant with creeping rhizomes, rufous branches with a tight cluster of punctures, violet red flowers with single-whorled petals.
C9□	Ciguo	Pingyin City, Shandong Province	Creeping plant with bright green leaves, ravidous branches with a tight cluster of punctures, violet red flowers with single-whorled petals and prickly hips.
C10□	Tanghong	Pingyin City, Shandong Province	Ravidous main branches with a small quantity of punctures, red flowers with single-whorled petals.
C11□	Baojialiyahong	Pingyin City, Shandong Province	Tall and erect plant with ravidous main branches and small quantity of punctures, pink flowers with triple-whorled petals. Its young shoots are green.
C1□2	Kushui	Kushui City, Gansu Province	Gray main branches with a small quantity of punctures, deep violet red flowers with four-whorled petals. Its leaves are smaller than other cultivars.
C13□	Luogang	Luogang City, Guangdong Province	Ravidous main branches and small quantity of punctures, violet red flowers with seven-whorled petals.
C14□	Lengxiang	Shenyang City, Liaoning Province	Ravidous main branches with a small quantity of punctures, deep pink flowers with more five-whorled petals.
C15□	Shanxian	Shan City, Shandong Province	Ravidous main branches with a small quantity of punctures, violet red flowers with more seven-whorled petals.
C16□	Yanling	Yanling City, Henan Province	Brown main branches with a small quantity of punctures, violet red flowers with more six-whorled petals.
C17□	Qingxu	Qingxu City, Shanxi Province	Ravidous main branches with a small quantity of punctures, violet red flowers with more seven-whorled petals. Its leaves are flatter than ‘chongbanhong’.
C18□	Xiaoxian	Xiao City, Anhui Province	Ravidous main branches with a small quantity of punctures, violet red flowers with more seven-whorled petals.
C19□	Miaofengshan	Beijing	Ravidous main branches with a small quantity of punctures, violet red flowers with more seven-whorled petals. Its leaves are larger and flatter than ‘chongbanhong’.
C20□	Chongbanhong	Pingyin City, Shandong Province	Ravidous main branches with a small quantity of punctures, deep violet red flowers with more seven-whorled petals.

Table 1. Continues.

C21□	Tangzi	Pingyin City, Shandong Province	Brown main branches with a small quantity of punctures and large branching angle, deep violet red flowers with more six-whorled petals.
C22□	Tangbai	Pingyin City, Shandong Province	Gray main branches with a large quantity of punctures and large branching angle, white flowers with six-whorled petals.
C23□	Zizhi	Pingyin City, Shandong Province	Amaranthine branches, amaranthine flowers with more six-whorled petals, and multi-seasonal flowering, its young shoots are lack of punctures.
C24□	Fenzhongguan	Pingyin City, Shandong Province	Ravidous main branches with a large quantity of punctures, pink flowers with more six-whorled petals.
C25□	Hanbao	Pingyin City, Shandong Province	Low plant, gray main branches with a small quantity of punctures and large branching angle, deep violet red flowers with triple-whorled petals, its buds can't fully open all along.
C26□	Canxueyingxia	Pingyin City, Shandong Province	Tall plant, ravidous main branches with small quantity of punctures and small branching angle, white flowers with four-whorled petals. Its young shoots are green.
C27□	Fense	Pingyin City, Shandong Province	Gray main branches with small quantity of punctures, pink flowers with five-whorled petals.
C28□	Daguo	Pingyin City, Shandong Province	Brown main branches with a small quantity of punctures, violet red flowers with four-whorled petals. Its hips are larger than other cultivars.
C29□	Liangye	Pingyin City, Shandong Province	Brown main branches with a small quantity of punctures, deep violet red flowers with triple-whorled petals. Its leaves are bright green.
C30□	Tianehuang	Pingyin City, Shandong Province	Erect plant with ravidous main branches and small quantity of punctures, pink flowers with triple-whorled petals. Its young shoots are green.

(□), Cultivated *R. rugosa*; (▲) wild *R. rugosa*.

Table 2. Nucleic acid sequences of DNA adaptors and primers used in AFLP analysis.

Adaptor and primer	Nucleic acid sequence
EcoR I joint	5'-CTCGTAGACTGGTACC-3' 5'-AATTGGTACGCAGTCTAC-3'
Mse I joint	5'-GACGATGAGTCCTGAG-3' 5'-TACTCAGGACTCAT-3'
EcoR I preamplification primer	5'-GACTGCGTACCAATTCA-3'
Mse I preamplification primer	5'-GATGAGTCCTGAGTAAC-3'
EcoR I selective amplification primer	E-AAC E-AAG E-ACA E-ACT E-ACC E-ACG E-AGC E-AGG
Mse I selective amplification primer	M-CAA M-CAC M-CAG M-CAT M-CTA M-CTC M-CTG M-CTT

The amplification using each pair of primers yielded 177 bands, and the average percentage of polymorphic bands was 96.2% (Table 3). This demonstrated the abundant genetic diversity of *R. rugosa* and fully reflected the high efficiency of AFLP in detecting the genetic diversity of *R. rugosa* germplasm resources.

Different primers yielded varying total number of bands from amplification, number of polymorphic bands and percentage of polymorphic bands (Table 3). The amplification using the E-AAC/M-CAG combination of primers resulted in the largest total number of bands (186), the largest number of polymorphic bands (185), and the highest percentage of polymorphic bands (99.5%) (Figure 1). In contrast, the amplification using the E-AAG/M-CAC combination of primers yielded the lowest total number of bands (155), the lowest number of polymorphic bands (146) and the lowest percentage of polymorphic bands (94.2%). Although, different

Table 3. Polymorphism of AFLP bands obtained by selective amplification based on the 10 primer pairs.

S/N	Primer combination	Number of total bands amplified	Number of polymorphic bands	PPB (%)
1	E-AAC/ M-CAA	178	172	96.6
2	E-AAC/ M-CAC	170	161	94.7
3	E-AAC/ M-CAG	186	185	99.5
4	E-AAC/ M-CTC	184	176	95.7
5	E-AAC/ M-CTT	175	167	95.4
6	E-AAG/ M-CAA	181	175	96.7
7	E-AAG/ M-CAC	155	146	94.2
8	E-AAG/ M-CTG	183	176	96.2
9	E-ACT/ M-CAC	194	187	96.4
10	E-ACT/ M-CAG	165	159	96.4
Total		1771	1704	
Average		177.1	170.4	96.2

PPB: Percentage of polymorphic banks.

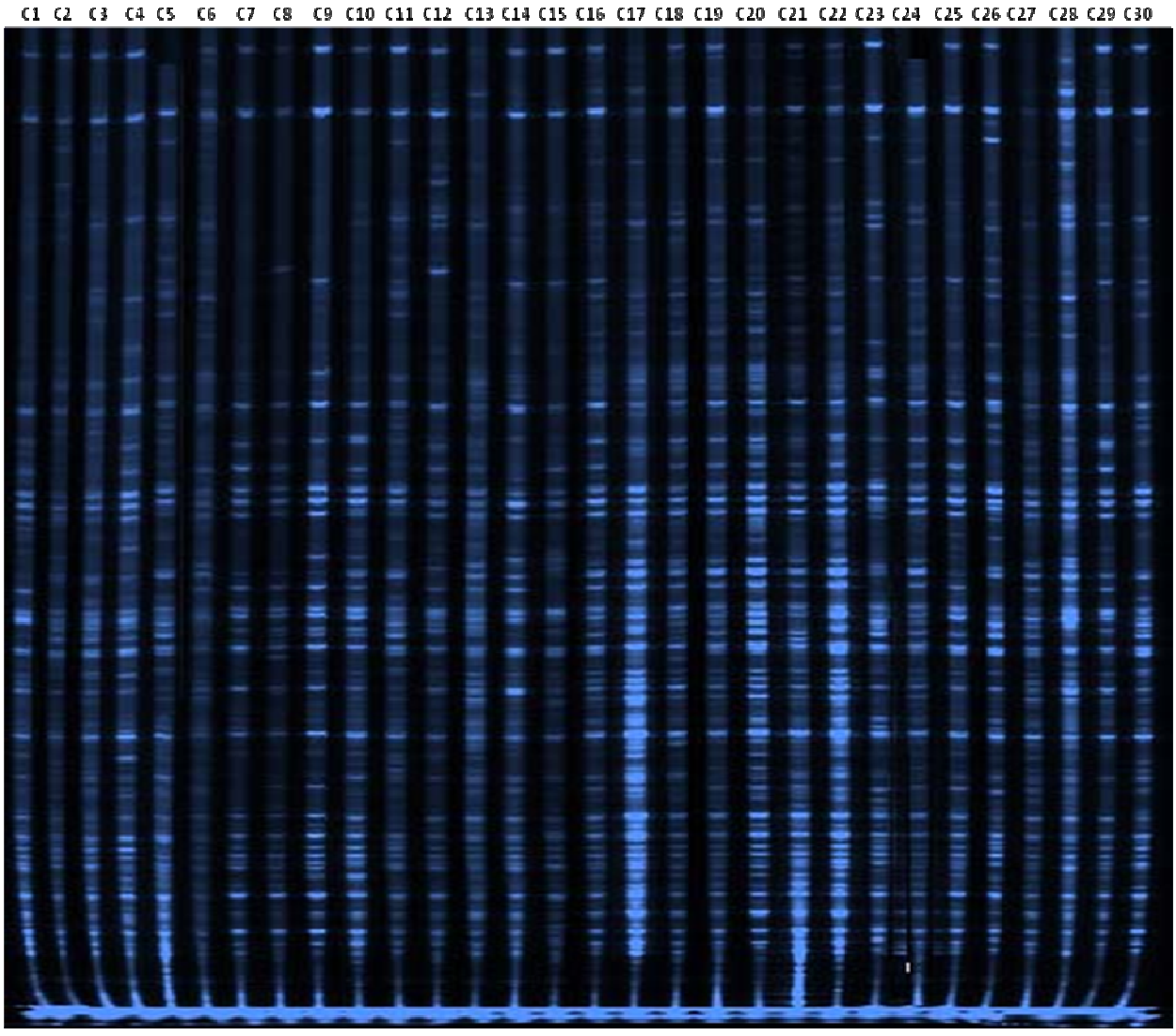

Figure 1. Amplified result of the 30 *R. rugosa* accessions by using primer combination E-AAC/M-CAG.

Table 4. Specific bands and identification percentage of primer combination for samples.

S/N	Primer combination	Specific allele (monomorphic)	Specific allele (null)	Identification percentage (%)
1	E-AAC/ M-CAA	22	6	53.3
2	E-AAC/ M-CAC	21	2	50.0
3	E-AAC/ M-CAG	22	3	33.3
4	E-AAC/ M-CTC	17	5	36.7
5	E-AAC/ M-CTT	23	6	56.7
6	E-AAG/ M-CAA	18	10	43.3
7	E-AAG/ M-CAC	15	5	40.0
8	E-AAG/ M-CTG	21	7	40.0
9	E-ACT/ M-CAC	19	5	33.3
10	E-ACT/ M-CAG	18	3	33.3
Average		19.6	5.2	42.00

primers produced different band quantities and types of polymorphisms from amplification, the percentage of polymorphic bands generated from each pair of primers was extremely high.

AFLP fingerprint specific alleles of *R. rugosa* accessions and identification efficiency of different primer combinations

The 10 pairs of primers detected varying numbers of specific alleles in the 30 *R. rugosa* accessions, including monomorphic alleles and null alleles (Table 4). The primer combination E-AAC/M-CTT produced the most monomorphic alleles (23), while E-AAG/M-CAA gene- rated the most null alleles (10). The highest identifi- cation efficiency was achieved by E-AAC/M-CTT (56.7%), but the identification efficiency of E-AAC/ M-CAA and E-AAC/M-CAC exceeded 50%. The primer combinations, E-AAC/M-CAA and E-AAC/M-CTT could identify a maximum of 24 accessions. Among the *R. rugosa* 30 accessions, 26 (except for 'Hunchun- yeshengI', 'Lengxiang', 'Yanling' and 'Miaofengshan') were found to have specific loci, accounting for 86.7% of all the accessions. The test materials were identified rapidly and accurately through these specific alleles (bands), thus providing a molecular basis for the classification and identification of *R. rugosa* germplasm resources.

Similarity analysis

The similarity coefficient of 30 *R. rugosa* accessions was between 0.4977 and 0.8410 (Figure 2) with an average of 0.6249. The indicated high similarity among the five wild *R. rugosa* was 0.6343, and the similarity coefficient between 'HunchunyeshengI' and 'HunchunyeshengII' was the largest (0.7732); this suggested low genetic variance between them because of the same origin. The similarity coefficients between 'Muping-yesheng', 'Rongchengyesheng', and 'Zhuangheyesheng' were smaller, and their average similarity coefficient was 0.5734, which indicated larger genetic difference and apparent genetic differentiation among them.

The average similarity coefficient of 25 *R. rugosa* cultivars was 0.6304 and the similarity coefficient between 'Qingxu' and 'Miaofengshan' was the largest (0.84100. The slightly high similarity coefficient between 'Luogang', 'Qingxu' and 'Chongbanhong' (above 0.79) suggested high similarity and low genetic difference between the three cultivars. On the other hand, the similarity coefficient between 'Baojialiyabai' and 'Kushui' was only 0.5089, which was indicative of high genetic difference. The similarity coefficient between 'Chong- banhong' and 'Tangbai' was 0.8077, while 'Ciguo' and 'Hanbao' reached 0.8054. The similarity coefficient between 'Zizhi' and 'Canxueyingxia' was also quite high; at 0.7582, which indicated that these accessions had high similarity and low genetic difference.

Clustering analysis

Figure 2 shows that the 30 *R. rugosa* accessions could be divided into two clusters with a similarity coefficient of 0.57. The first cluster included six accessions divided into two sub-clusters. The first sub-cluster had only one accession (C2), while the second sub-cluster had five accessions divided into two groups: the first with three accessions (C3, C30 and C11) and the second with two accessions (C23 and C26).

The second cluster included 24 accessions divided into three sub-clusters. The first sub-cluster had 11 accessions classified into five groups: the first with only

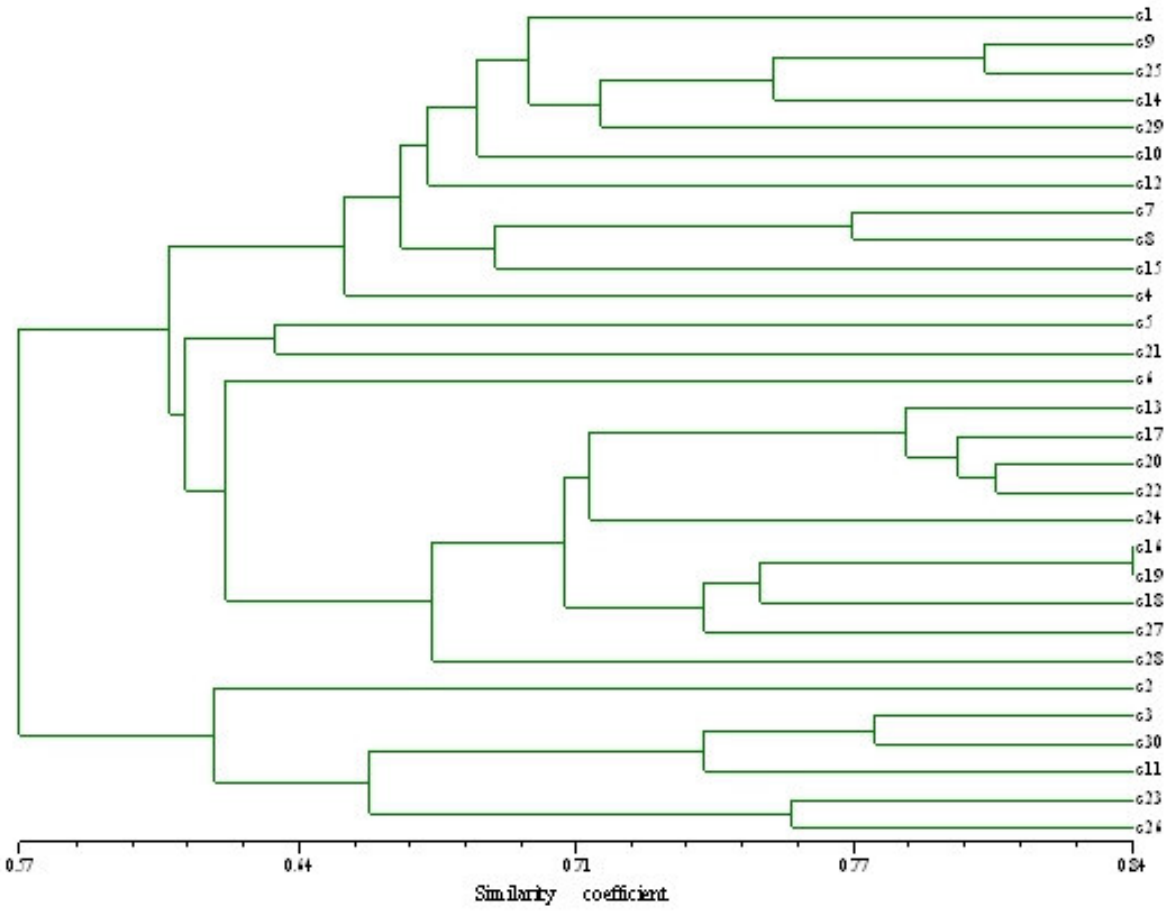

Figure 2. Dendrogram of 30 *R. rugosa* accessions based on AFLP analysis with 10 pair primers combinations.

one accession (C4); the second with three accessions (C7, C8 and C15; the third and fourth with one accession each, C12 and C10, respectively); and the fifth with five accessions (C1, C9, C25, C14 and C29). The second sub-cluster had two accessions (C5 and C21). The third sub-cluster had 11 accessions which was divided into five groups: the first with one accession (C6); the second with four accessions (C13, C17, C20 and C22); the third with one accession (C24); the fourth with four accessions (C16, C19, C18 and C27); and the fifth with one accession (C28).

DISCUSSION

A greater genetic diversity at the DNA molecular level implies a longer evolutionary history of the species (Li et al., 2005). This study analyzed the genetic differences of 30 *R. rugosa* germplasm accessions using the fluorescent marker AFLP method and obtained 96.2% polymorphic locus, which revealed the extreme abundance of genetic diversity in the Chinese *R. rugosa* germplasm resources. *R. rugosa* was reported to be completely self-incompatible (Bruun, 2005; Yu et al., 2009), so the high genetic diversity could mainly come from gene flow during the course of hybridization and genetic evolution.

The similarity coefficient of the 30 *R. rugosa* accessions was between 0.4977 and 0.8410, with an average of 0.6249; this indicated high similarity and low genetic difference between the *R. rugosa* germplasm resources in China. Many *R. rugosa* accessions had similar morphological features (Yang et al., 2003; Yu et al., 2005; Li, 2006), which were possibly responsible for the confusion of homonyms and synonyms in *R. rugosa* germplasm resources today. In summary, combined with our previous study (Feng et al., 2009), we found that there were low average similarity coefficient and remote relationships between the wild and cultivated accession of *R. rugosa* in China.

Clustering results (Figure 2) revealed that the first cluster included ‘Baojialiyabai’, ‘Sulianxiangshui’, ‘Baojialiyahong’, ‘Canxueyingxia’, ‘Tianehuang’ and ‘Zizhi’. The average similarity coefficient between these five and other 27 *R. rugosa* accessions were also smaller, and their genetic difference was higher too.

Previous studies found that the five accessions had apparent differences in the morphological characteristics against the traditional Chinese *R. rugosa* cultivars; their morphological features were more similar to *Rosa damascena* or *Rosa centifolia* (Yang et al., 2003; Li, 2006), indicating that they were possibly *R. damascena* or *R. centifolia* or its hybrids with traditional Chinese *R. rugosa* cultivars. 'Zizhi' was a hybrid of *Rosa davurica* Pall. and *Rosa rugosa cv.* "plena" (Tang, 1994), making their morphological and biological features quite different from the traditional Chinese *R. rugosa* cultivars. The second cluster included exclusively wild accessions and the current major cultivars originated from China, and they were all traditional Chinese *R. rugosa*.

'Chongbanhong', 'Yanling', 'Miaofengshan', 'Xiaoxian', 'Luogang' and 'Qingxu' were major cultivars of the *R. rugosa* production areas in China. The results of this study revealed that the average similarity coefficient of these six accessions was 0.7428, which indicated high similarity and low genetic difference among them, in which, 'Qingxu' and 'Miaofengshan' had the largest similarity coefficient (0.8410) and the lowest genetic difference under the same sub-cluster. 'Luogang', 'Qingxu' and 'Chongbanhong' belonged to the same group and were closely genetically related to each other, whereas 'Yanling', 'Miaofengshan' and 'Xiaoxian' were in the same group and had close genetic relationship. These indicated that the main cultivated *R. rugosa* in China had high similarity, low genetic difference and narrow genetic background, which were possibly responsible for the difficulty of breakthroughs in Chinese *R. rugosa* breeding. Therefore, we recommend the rapid introduction of fine foreign *R. rugosa* germplasm resources, especially wild accessions, to improve the traditional *R. rugosa* cultivars in China, enrich genetic basis, and breed fine and new cultivars as early as possible.

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