Genetic Diversity of Pummelo Germplasms in Sichuan Basin Based on RAPD Markers

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Abstract. Sichuan Basin is one of the centers of pummelo cultivation, domestication and diversity. Also, as one of the main production areas, Sichuan Basin possessed abundant pummelo germplasm resources. In this study, genetic diversity analysis among 73 accessions of pummelo (Citrus grandis Osbeck) and rootstocks from Sichuan Basin was assessed by using RAPD markers. A total of 156 bands were amplified by 15 RAPD primers, 93.59% (146) of which 146 were polymorphic with an average of 10.4 polymorphic bands per primer. The genetic similarity coefficients of 73 germplasms varied from 0.4511 to 0.9808 at the accession level. At the group level, the maximum GS coefficient index was observed between Wendan and Guanxi Miyou & bud mutants (0.8838), and the minimum GS coefficient was shown in Shatianyou and Putao you (0.6852). 73 accessions of pummelo and rootstocks were clustered into five groups with UPGMA method at distance coefficient of 0.6102, consisting of Shatian pummelo varieties group, Wendan pummelo varieties group, Guanxi Miyou & bud mutants group, Putao pummelo varieties group, and rootstocks of C. junos (Pujiang Xiangcheng and Ziyang Xiangcheng) and Poncirus trifoliata. In addition, Guanxi Miyou and bud mutants were clearly separated in our RAPD analysis. This study demonstrated abundant genetic variation among Guanxi Miyou and bud mutants, which could be used core germplasms in the future pummelo breeding program.

1. Introduction
Pummelo, Citrus grandis (L.) osbeck, belongs to genus Citrus L., family Rutaceae [1]. China is one of major centers of origination and genetic diversity, possessing abundant pummelo germplasms. The cultivation history of pummelo can be date back to 3000 years ago [2], including three major cultivation areas, Southeast coastal, South China and Southwest China [3]. Until now, more than 200 pummelo varieties have been selected and cultivated in China [4]. As important pummelo production area in Southwest China, Sichuan Basin accounts for one third of cultivation area in China. There are abundant pummelo varieties, such as Cuixiantian pummelo, Guanxi Miyou, Shatian pummelo, Liangping pummelo and so on [3]. However, genetic diversity among pummelo germplasms in Sichuan Basin remains unclear, especially the origin of local germplasms and genetic relationships among them.

RAPD, random amplification of polymorphic DNA, as dominant markers, has been used to analyze the genetic diversity among pummelo germplasms [5]. To provide basis for pummelo breeding and
resource conservation, we carried out genetic diversity among pummelo germplasms and rootstocks from Sichuan Basin by using both RAPD and SSR markers.

2. Materials and methods

2.1. Plant materials
We sampled seventy pummelo germplasms and three rootstocks from Sichuan, Fujian Provinces and Chongqing city. The cultivar name and source are listed in the Table 1.

Table 1. The pummelo germplasms and rootstocks were used in this study

| Code | Cultivars       | Source                      | Code | Cultivars       | Source                      |
|------|-----------------|-----------------------------|------|-----------------|-----------------------------|
| 1    | Hongrou Miyou 1 | Langzhong, Sichuan          | 38   | Xujia Baiyou 1  | Dianjiang, Chongqing        |
| 2    | Huangjin Miyou 1| Langzhong, Sichuan          | 39   | Xujia Baiyou 2  | Dianjiang, Chongqing        |
| 3    | Hongman Miyou 1 | Langzhong, Sichuan          | 40   | Xujia Baiyou 3  | Dianjiang, Chongqing        |
| 4    | Sanhong Miyou 1 | Langzhong, Sichuan          | 41   | Suanyou         | Dianjiang, Chongqing        |
| 5    | Guanxi Miyou 1  | Langzhong, Sichuan          | 42   | Jintang Wuheyou | Jintang, Sichuan            |
| 6    | Guanxi Miyou 2  | Puijiang, Sichuan           | 43   | Guokuiyou       | Jintang, Sichuan            |
| 7    | Hongrou Miyou 2 | Puijiang, Sichuan           | 44   | Jintang Lvyou   | Jintang, Sichuan            |
| 8    | Huangjin Miyou 2| Puijiang, Sichuan           | 45   | Meiwanyou       | Leshan, Sichuan             |
| 9    | Hongman Miyou 2 | Puijiang, Sichuan           | 46   | Fenghuangyou    | Daxian, Sichuan             |
| 10   | Sanhong Miyou 2 | Puijiang, Sichuan           | 47   | Bingtangyou     | Shehong, Sichuan            |
| 11   | Hongjin Miyou 3 | Pinghe, Fujian              | 48   | Yong'anyou      | Zhongjiang, Sichuan         |
| 12   | Hongrou Miyou 3 | Pinghe, Fujian              | 49   | Pengxian Xianluoyou | Pengxian, Sichuan      |
| 13   | Sanhong Miyou 3 | Pinghe, Fujian              | 50   | Hejiangyou      | Hejiang, Sichuan            |
| 14   | Huangjin Miyou 3| Pinghe, Fujian              | 51   | Kuyou           | Jingyan, Sichuan            |
| 15   | Poncirus trifoliata| Pujiang, Sichuan          | 52   | Tongxianyou     | Neijiang, Sichuan           |
| 16   | C. junos cv. 'Ziyang Xiangcheng' | Pujiang, Sichuan     | 53   | Long'anyou      | Guang'an, Sichuan           |
| 17   | C. junos cv. 'Pujiang Xiangcheng' | Pujiang, Sichuan     | 54   | Shuijing Wendan | Sichuan                    |
| 18*  | Dongguaqian Shatianyou | Changshou, Chongqing | 55*  | Taiji Tuyou     | Jintang, Sichuan            |
| 19*  | Gulaooqian Shatianyou | Changshou, Chongqing | 56*  | Huayingshan     | Guang'an, Sichuan           |
| 20   | Beibeiyou       | Beibei, Chongqing           | 57   | Aiwanyou        | Sichuan                    |
| 21*  | Linnan Shatianyou| Jiangjin, Chongqing        | 58   | Pingshanyou     | Hu'an, Fujian               |
| 22*  | Juhuaxin Shatianyou | Changshou, Chongqing      | 59   | Hangwan Miyou   | Longyan, Fujian             |
| 23   | Waneiyou        | Jiangjin, Chongqing        | 60   | Fujian Wendan   | Hu'an, Fujian               |
| 24   | Anjiangyou      | Jiangjin, Chongqing        | 61*  | Humiyou         | Liangping, Sichuan          |
| 25   | Wubu Hongxinyou | Ba'nan, Chongqing          | 62*  | Jinshayou       | Xin'gan, Jiangxi            |
| 26   | Pengxiyou       | Jiangjin, Chongqing        | 63*  | Zhaipoyou       | Nankang, Jiangxi            |
| 27   | Cuixiang Tianyou| Nanchong, Sichuan          | 64*  | Jiangxi Zaoyou  | Jiangxi                     |
| 28   | Guanxiangyou    | Beibei, Chongqing          | 65*  | Maohuahong      | Nankang, Jiangxi            |
| 29   | Naxi Yingtaoyou | Beibei, Chongqing          | 66*  | Yuenan Xiaoyou  | Xishuangbanna, Yunnan      |
| 30   | Dianjiang Zenjia Baiyou | Jiangjin, Chongqing       | 67*  | Dongfeng Zaoyou | Xishuangbanna, Yunnan      |
| 31   | Duanshiyou      | Jiangjin, Chongqing        | 68   | Huyou           | Huangyan, Zhejiang          |
| 32   | Dianjiang Hongxinyou | Dianjiang, Chongqing     | 69*  | Shijie Miyou   | Zhejiang                    |
| 33   | Liangpingyou    | Dianjiang, Chongqing       | 70*  | Anjiang Hongxinyou | Anjiang, Hu'nan |
| 34   | Fengdu Sanyuan  | Dianjiang, Chongqing       | 71   | Huoyan          | America                     |
| 35   | Rentou Dahongyou | Dianjiang, Chongqing       | 72*  | Ruihong Putaoyou | Califorlia, America      |
| 36   | Zhoujia Baiyou  | Dianjiang, Chongqing       | 73*  | Jiwei Putaoyou  | Califorlia, America        |
| 37   | Dianjiang Baiyou | Dianjiang, Chongqing       |      |                 |                             |

Note: * represents the germplasms from Citrus Research Institute, Chinese Academy of Agricultural Sciences (Chongqing, China).
2.2. **DNA extraction and PCR amplification**

Total genomic DNA was isolated from silica-gel dried leaf tissues using a modified CTAB method [6]. 15 RAPD primers were selected out from 20 primers through pre-optimization tests [7]. Those primers exhibiting high polymorphism and good reproducibility were further used to screen the full set of accessions (Table 2). PCR amplification was followed by Ji et al. [8], which was performed in a 25 μL volume, containing 20 ng of genomic DNA, 1.2 μL of MgCl₂ (25 mmol•L⁻¹), 1.4 μL of dNTP mix (10 mmol•L⁻¹), 1 μL of each primer (5 μmol•L⁻¹), 1.5 U of PfuDNA polymerase (Tiangen, Beijing), and 2.0 μL of 10× PCR buffer (10 mmol•L⁻¹ pH 8.0 Tris-HCl, 50 mmol•L⁻¹ KCl, 1.5 mmol•L⁻¹ EDTA). Conditions for amplification consisted of an initial denaturation at 94°C for 4 min, followed by 45 cycles at 94°C for 1 min, then at 36°C for 1 min and at 72°C for 2 min, with a final extension at 72°C for 10 min. Amplifications were carried out using a PTC-200 thermocycler (Bio-rad, Hercules, CA). The PCR products were separated in a 1.5% agarose gel containing 0.5 μg•mL⁻¹ EiBr. The bands were then visualized under UV light and photographed.

2.3. **Data analysis**

The electropherogram results were presented as a binary matrix, consisting of ‘1’ for the presence and ‘0’ for the absence of the amplicons at the same locus. A dendrogram was constructed by following UPGMA (unweighted paired group method using arithmetic averages) option of SAHN (sequential, agglomerative, hierarchical and nested) module in NTSYS software package version 2.2 [9]. Genetic distances of Euclidan's simple matching coefficient measure (GD₅₆₉₅) was estimated as: \( GD₅₆₉₅ = 1 - \frac{(N₁₁+N₀₀)}{(N₁₁+N₁₀+N₀₁+N₀₀)} \), where \( N₁₁, N₀₀, N₁₀, \) and \( N₀₁ \) were the number of band alleles common to both individuals, absence in both individuals; specific to individuals \( i \) or \( j \), respectively. \( N \) represented the total number of band alleles [10].

3. Results

3.1. **Polymorphic Analysis**

Among 20 primers tested, 15 RAPD primers selected for the analysis generated polymorphic allelic patterns. RAPD analysis of 73 genotypes yielded 156 fragments, 93.59% (146) of which were polymorphic with an average of 10.4 polymorphic fragments per primer. Maximum polymorphism was observed in the amplification pattern by primer R-222 (Table 2). Each primer could accurately differentiate 73 pummelo germplasms and rootstocks (Figure 1).

Table 2. Polymorphism comparison of amplified products of 73 pummelo germplasms and rootstocks amplified by RAPD primers

| Primers | Sequence (5’-3’) | Total number of amplified bands | Number of polymorphic bands | Percentage of polymorphism (%) |
|---------|-----------------|--------------------------------|----------------------------|--------------------------------|
| R-212   | GTGACGTAGG      | 6                              | 6                          | 100.00                         |
| R-213   | GTGATCGCAG      | 11                             | 10                         | 90.91                          |
| R-216   | AGCCAGACGA      | 11                             | 9                          | 81.82                          |
| R-217   | GGTGATCGAG      | 13                             | 12                         | 92.31                          |
| R-218   | GTGCTTAACC      | 12                             | 12                         | 100.00                         |
| R-220   | AATCGGGGCTG     | 10                             | 10                         | 100.00                         |
| R-221   | GGCACTGAGG      | 11                             | 9                          | 81.82                          |
| R-222   | GAGGCTTCCA      | 17                             | 16                         | 94.12                          |
| R-223   | GTCAGGGGCA      | 13                             | 13                         | 100.00                         |
| R-225   | GGTAGCAGTC      | 8                              | 7                          | 87.50                          |
| R-226   | GGTTCCTCAG      | 9                              | 8                          | 88.89                          |
| R-227   | TCAGGATGGC      | 12                             | 12                         | 100.00                         |
| R-228   | ACTCAGAGAG      | 8                              | 8                          | 100.00                         |
| R-231   | CAGTTCAGAG      | 8                              | 7                          | 87.50                          |
| R-232   | AGCCAGCGAA      | 7                              | 7                          | 100.00                         |
| Total   |                 | 156                            | 146                        | 93.59                          |
3.2. Clustering analysis based on similarity coefficient

On the basis of molecular data, a matrix of genetic similarity (GS) coefficient was computed following the procedure of Nei [11]. The similarity coefficient based on 15 RAPD amplicons ranged from 0.4511 to 0.9808 at the accession level. At the group level, the maximum GS coefficient index was observed between Wendan pummelo varieties group and Guanxi Miyou & bud mutants (0.8838), and the minimum GS coefficient was shown in Shatian pummelo varieties group and Putao pummelo varieties group (0.6852) (Table 3).

A dendrogram of the genetic relationships among pummelo germplasms and rootstocks was drawn using GS values according to the UPGMA method. The results indicated that these accessions were divided into five major groups, consisting of Shatian pummelo varieties (A), Wendan pummelo varieties (Guanxi Miyou & bud mutants) (B), Putao pummelo varieties (C), and rootstocks of Citrus junos (D) and Poncirus trifoliata (E) (Figure 2). Group B was the most complicated, including most accessions, with Wendan pummelo varieties, Guanxi Miyou & bud mutants, and several hybrid pummelo (such as Kuiyou).

Table 3. Genetic distance and similarity index among pummelo populations based on RAPD markers

| Genetic similarity distance | Genetic | Shatianyou | Wendanyou | Guanxi Miyou & bud mutants | Putaoyou | Rootstocks |
|----------------------------|---------|------------|-----------|---------------------------|----------|------------|
| Shatianyou                 | ****    | 0.8460     | 0.7395    | 0.6852                    | 0.6767   |            |
| Wendanyou                  | 0.1673  | ****       | 0.8838    | 0.7813                    | 0.7608   |            |
| Guanxi Miyou & bud mutants | 0.3018  | 0.1273     | ****      | 0.7319                    | 0.7177   |            |
| Putaoyou                   | 0.3780  | 0.2467     | 0.3122    | ****                      | 0.6981   |            |
| Rootstocks                 | 0.3905  | 0.2733     | 0.3317    | 0.3594                    | ****     |            |

4. Discussion

Pummelo (C. grandis) is a monomorphic plant. During the long natural selection and artificial domestication history, there are various embryo mutants and natural hybrids with abundant genetic diversity. Based on morphological characters, pummelo varieties could be divided into Shatian pummelo, Wendan pummelo, and hybrid pummelo groups by He et al. [3]. In this study, most Shantian pummelo varieties were clustered into group A, while Wendan pummelo varieties were scattered into several subgroups. This might be related that Wendan pummelo varieties could be divided into true Wendan pummelo varieties and common pummelo varieties. This was also consistent with the morphological characters [12]. Also, we obtained incongruent results between cluster and morphology, such as Duanshi pummelo. According to traditional taxonomy, it is regarded as bud mutants of Shatian pummelo, while it is clustered in the Wendan pummelo varieties group. Liu et al. [12] supposed that gene flow was occurred between Duanshi pummelo and Wendan pummelo during the long cultivation history. More powerful evidence will be needed to confirm this hypothesis.

In this study, RAPD markers provided abundant genetic variation among pummelo germplasms and rootstocks. The UPGMA cluster analysis through RAPD markers could differentiate pummelo
germplasms and evaluate the genetic relationships among pummelo germplasms. Shi [13] suggested that RAPD markers are effective tools for identification and genetic relationships of pummelo varieties. In addition, the genetic relationships among Guanxi Miyou & bud mutants were demonstrated by RAPD markers, indicating abundant genetic variation among them. Therefore, we could consider Guanxi Miyou & bud mutants, originated from Fujian Province, as the core germplasm of pummelo, which will provide abundant information for pummelo cultivation program in the future.

Figure 2. Clustering analysis of 73 pummelo germplasms and rootstocks based on RAPD markers

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References
[1] Huang, C.J. (1997) Flora of China. Science Press, Beijing.
[2] Ye, Y.M. (1997) The diversity center of pummelo germplasm. South China Fruits, 26(1): 3–5.
[3] He, T.F. (1999) Grapefruit cultivation in China. China Agricultural Press, Beijing.
[4] Deng, X.X., Peng, S.A. (2013) Citrus. China Agricultural Press, Beijing.
[5] Zhang, T.P., Peng, S.L., Wang, Z.F., Ling, D.H., Gan, L.S. (2001) Genetic relationships among cultivars of *Citrus maxima* (Burm.) merr. using RAPD marker technique. J. Tropic. Subtropic. Bot. 9(4): 322–328
[6] Zhou, Y.Q. (2005) Application on DNA molecular marker technology in plant study. Chemical Industry Press, Beijing.
[7] Sankar, T.G., Gopi, V., Deepa, B., Gopal, K. (2014) Genetic diversity analysis of sweet orange (*Citrus sinensis* osbeck) varieties / clones through RAPD markers. Int. J. Curr. Microbiol. App. Sci., 3(4): 75–84.
[8] Ji, Q.H., Zeng, J.W., Guo, Y.J. (2013) Using optimized random amplified polymorphic DNA (RAPD) markers to identify the category status of *Citrus nobilis* Lour. Gonggan. Afr. J. Biotechnol., 10(64): 13982–13990.
[9] Rohlf, F.J. (2000) NTSYS-pc numerical taxonomy and multivariate analysis system version 2.1 [OL]. Applied Biostatistics Inc, New York.
[10] Sokal, R.R., Michener, C.D. (1958) A statistical method evaluating systematic relationships. Univ. Kans. Sci. Bull., 38: 1409–1438.
[11] Nei, M. (1978) Estimation of average heterozygostity and genetic distance from a small number of individuals. Genetics, 89: 583.
[12] Liu, Y., Sun, Z.H., Liu, D.C., Wu, B., Tao, J.J. (2005) Assessment of the genetic diversity of pummelo germplasms using AFLP and SSR markers. Scientia Agricultura Sinica, 38(11): 2308–2315.
[13] Shi, K.M. (2004) Genetic diversity of pummelo germplasm resources in western Hubei based on RAPD and ISSR markers. Huazhong Agricultural University, Wuhan.