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Genetic Diversity of Pummelo (*Citrus grandis* Osbeck) Germplasms in Sichuan Basin Inferred from SSR Markers

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Abstract. Sichuan Basin is one of centers of the main distribution area of pummelo (*Citrus grandis* Osbeck) genetic germplasms. It is necessary to analyze the genetic relationship of the local germplasms and to get enough information for protecting and utilizing them in the future. In this study, fifteen simple sequence repeat (SSR) primers mined in Citrus were used to detected the genetic diversity of 73 pummelo germplasms and rootstocks collected in Sichuan Basin. A total of 123 alleles were generated across 15 SSR loci, with an average of 8.2 alleles per locus. The observed heterozygosity (H_o) ranged from 0.0000 to 0.7429 with an average 0.2090 per locus, while expected heterozygosity (H_e) varied from 0.0273 to 0.6068 with an average of 0.2395 per locus. Obvious differences of genetic diversity were observed among different pummelo varieties groups. The average genetic diversity index among groups showed as follows: N_a = 1.6935, H = 1.4404, I = 0.3363, H_o = 0.2570, H_e = 0.2470. At the group level, rootstocks of *C. jonus* and *Poncirus trifoliata* revealed the highest genetic diversity index (H = 1.9685, I = 0.6854, H_o = 0.3556, H_e = 0.5244), and Shatian pummelo varieties showed the lowest index (H = 1.2486, I = 0.2202, H_o = 0.1611, H_e = 0.1590). The highest genetic similarity coefficient (0.9668) was observed between Guanxi Miyou & bud mutants group, and Wendan pummelo varieties group, and the lowest one (0.7831) was detected between Guanxi Miyou & bud mutants group, and Putao pummelo varieties group (*C. paradisi*). 73 accessions of pummelo and rootstocks were clustered into five groups with UPGMA method, 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*. No significant differentiation was observed between Guanxi Miyou and bud mutants by SSR markers.

INTRODUCTION

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 Cuixiang Tianyou, Guanxi Miyou, Shatianyou, Liangpingyou 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.

Simple sequence repeats (SSR) assay has also been a powerful molecular method for assessing genetic variation in plant due to its low cost, high polymorphism, co-dominant inheritance [5, 6]. To provide basis for pummelo breeding and resource conservation, we carried out genetic diversity among pummelo germplasms and rootstocks from Sichuan Basin by using SSR markers.

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MATERIALS AND METHODS

Plant Materials

We sampled 70 pummelo germplasms and three rootstocks from Sichuan, Fujian Provinces and Chongqing city. The cultivar name and source are listed in 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	Hongmian 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	Pujiang, Sichuan	43	Guokuiyou	Jintang, Sichuan
7	Hongrou Miyou 2	Pujiang, Sichuan	44	Jintang Lvyou	Jintang, Sichuan
8	Huangjin Miyou 2	Pujiang, Sichuan	45	Meiwanyou	Leshan, Sichuan
9	Hongmian Miyou 2	Pujiang, Sichuan	46	Fenghuangyou	Daxian, Sichuan
10	Sanhong Miyou 2	Pujiang, Sichuan	47	Bingtangyou	Shehong, Sichaun
11	Hongmian 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	Kuiyou	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*	Dongguaquan Shatianyou	Changshou, Chongqing	55*	Taiji Tuyou	Jintang, Sichuan
19*	Gulaoqian Shatianyou	Changshou, Chongqing	56	Huayingshan Xiangyou	Guang'an, Sichuan
20	Beibeiyou	Beibei, Chongqing	57	Aiwanyou	Sichuan
21*	Linnan Shatianyou	Jiangjin, Chongqing	58	Pingshanyou	Hua'an, Fujian
22*	Juhuaxin Shatianyou	Changshou, Chongqing	59	Hangwan Miyou	Longyan, Fujian
23	Wanbeiyou	Jiangjin, Chongqing	60	Fujian Wendan	Hua'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 Hongxinyou	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).

DNA Extraction and PCR Amplification

Total genomic DNA was isolated from silica-gel dried leaf tissues using a modified CTAB method [7].

15 pairs of SSR primers were selected out from 30 pairs of primers through pre-optimization tests [8]. 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 Jiang et al. [9], which was performed in a 20 μL volume, containing 1 μL of genomic DNA, 7 μL of ddH_2O, 1 μL of the forward and reverse primer, and 10 μL of Taqmix (Tiangen, Beijing). The amplification conditions consisted of an initial denaturation at 94°C for 4 min, followed by 34 cycles at 94°C for 50 s, then at 56°C for 1 min and at 72°C for 1.5 min, with a final extension at 72°C for 8 min. The PCR products were separated in 8% denaturing polycrylamide gel for two to three hours depending on the amplican size. Finally, the DNA bands were visualized using the silver staining method [10]. If the first PCR failed to give an amplification product, the PCR was repeated.

TABLE 2. Amplified results and polymorphism information of SSR primers

Primers	Sequence (5'-3')	Observed heterozygosity (H_o)	Nei's Excepted heterozygosity (H_e)
C-3	F: CACAGCTAAAGCCAGCACAA R: GAAGAGACAGCGGGTAGCAC	0.0556	0.0812
C-5	F: CAGCGTCGCTTATCACAAAG R: CCACAATGCACAATGAGGAG	0.0137	0.0273
C-12	F: AAGAAGAGGAGCCCCCATTA R: CTGGCAACGACAACATCAAC	0.7429	0.5229
C-17	F: GCATTACGAGCTGCAGTTGA R: ATGAACAAAGCATCCCATCG	0.0411	0.0672
C-18	F: CAGCGACAACAAAAGCAAGA R: CTTGGATCGAGACATCAGCA	0.3056	0.371
C-21	F: GAAACTTCCCACTTCGCTCA R: TTTCGGACCATCCAAAACTC	0.0278	0.1057
M-41	F: GCCATGACGGATGAAGAGAT R: AGCGACGTATCGATCAGCTT	0.0000	0.0801
M-52	F: CTTCTTGACGAGTGCTGCTG R: CAAGTTCATGCTTCAGGCAA	0.0274	0.0537
M-57	F: GATCCATGCAGGAGCTTGTT R: CTTGATTCCCCTTTCAACGA	0.0299	0.0871
M-81	F: AAATGACGTTCAAGCCTTCG R: TCGAAAGTACCAAAATGGGC	0.4638	0.5635
M-86	F: AATGCCACAAAACTGTCGGT R: TTGCAAGAGGAGACTTGCCT	0.0274	0.0801
M-87	F: GGGAAAGCCCTAATCTCATCA R: CTTCCTCTTGCGGAGTGTTC	0.3014	0.6068
M-89	F: TCGTCACACCATTTTGTTGAA R: ACGGCAGATTTGAGCTGAGT	0.0556	0.0547
M-91	F: TGTGAGTGCTCTTCACCGTC R: CAATCTCAAACACGAAAACCAA	0.5634	0.4195
M-93	F: GCCGAGAAATTGCGAGAA R: CGTATACCACCGCCTTCATT	0.4795	0.4713
Mean		0.2090	0.2395

Data Analysis

We employed the Quantity One software (Biorad, USA) to approximate the band size via referring to the standard molecular weight size marker (20 bp DNA ladder, Takara), and then converted these size-based data into different formats in GenALEx 6.5 [11] for further analysis. The genotypic data were used to estimate genetic diversity. The number of alleles (N_a), effective number of alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e) and allele frequency were calculated with GenALEx 6.5 software. 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 [12].

RESULTS

PCR Amplification and Polymorphic Analysis

Among 30 pairs of primers examined, 15 pairs selected for the analysis generated polymorphic allelic patterns. A total of 123 alleles were generated across 15 SSR loci, with an average of 8.2 alleles per locus. The observed heterozygosity (H_o) ranged from 0.0000 (M-41) to 0.7429 (C-12) with an average 0.2090 per locus, while expected heterozygosity (H_e) varied from 0.0273 (C-5) to 0.6068 (M-87) with an average of 0.2395 per locus (Table 2). This suggested obvious differences among different SSR loci. Each pair of primers could differentiate pummelo germplasms and rootstocks (Fig. 1).

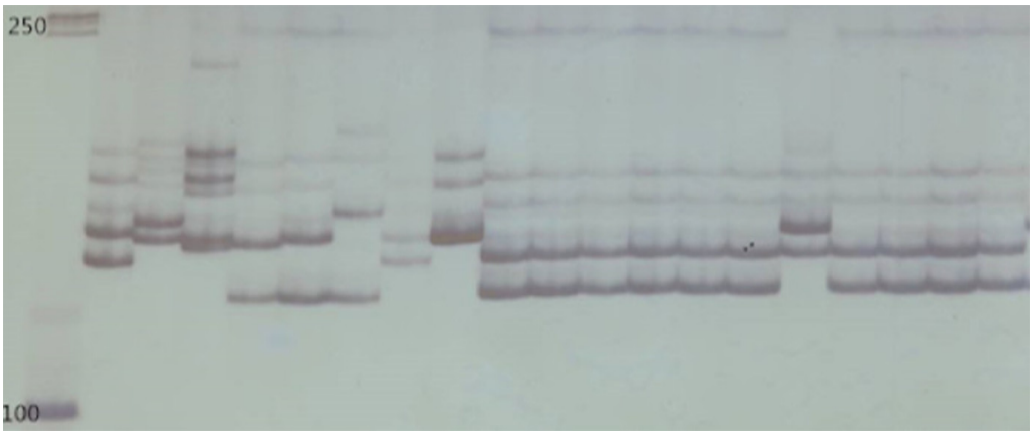

FIGURE 1. The PCR amplification for some pummelo germplasms by SSR primers

As shown in Table 3, obvious differences of genetic diversity were observed among different pummelo varieties groups. The average genetic diversity index among groups showed as follows: N_a = 1.6935, H = 1.4404, I = 0.3363, H_o = 0.2570, H_e = 0.2470). At the group level, rootstocks revealed the highest genetic diversity index (H = 1.9685, I = 0.6854, H_o = 0.3556, H_e = 0.5244), and Shatian pummelo varieties showed the lowest index (H = 1.2486, I = 0.2202, H_o = 0.1611, H_e = 0.1590).

TABLE 3. Genetic diversity among pummelo populations based on SSR markers

Populations	Observed number of alleles (N_a)	Genetic diversity index (H)	Shannon's information index (I)	Observed heterozygosity (H_o)	Nei's excepted heterozygosity (H_e)
Shatianyou	1.4667	1.2486	0.2202	0.1611	0.1590
Wendanyou	2.0000	1.3618	0.3102	0.1698	0.1856
Guanxi Miyou & bud mutants	1.4000	1.3163	0.2422	0.3095	0.1748
Putaoyou	1.3333	1.3067	0.2235	0.2889	0.1911
Rootstocks	2.2677	1.9685	0.6854	0.3556	0.5244
Mean	1.6935	1.4404	0.3363	0.2570	0.2470

Clustering Analysis Based on Similarity Coefficient

The similarity coefficient based on 15 pairs of SSR amplicons ranged from 0.7831 to 0.9668 at the group level. The maximum GS coefficient index was observed between Wendan pummelo varieties group and Guanxi Miyou & bud mutants, and the minimum GS coefficient was shown in Guanxi Miyou & bud mutants, and Putao pummelo varieties group (0.6852) (Table 4).

TABLE 4. Genetic distance and similarity index among pummelo populations based on SSR markers

Genetic similarity / Genetic distance	Shatianyou	Wendanyou	Guanxi Miyou & bud mutants	Putaoyou	Rootstocks
Shatianyou	***	0.9611	0.9248	0.7976	0.5162
Wendanyou	0.0397	***	0.9668	0.8312	0.5413
Guanxi Miyou & bud mutants	0.0782	0.0338	***	0.7831	0.4959
Putaoyou	0.2262	0.1848	0.2245	***	0.7471
Rootstocks	0.6612	0.6137	0.7014	0.4737	***

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, Wendan pummelo varieties (Guanxi Miyou & bud mutants), Putao pummelo varieties, and rootstocks of *Citrus junos* and *Poncirus trifoliata* (Fig. 2).

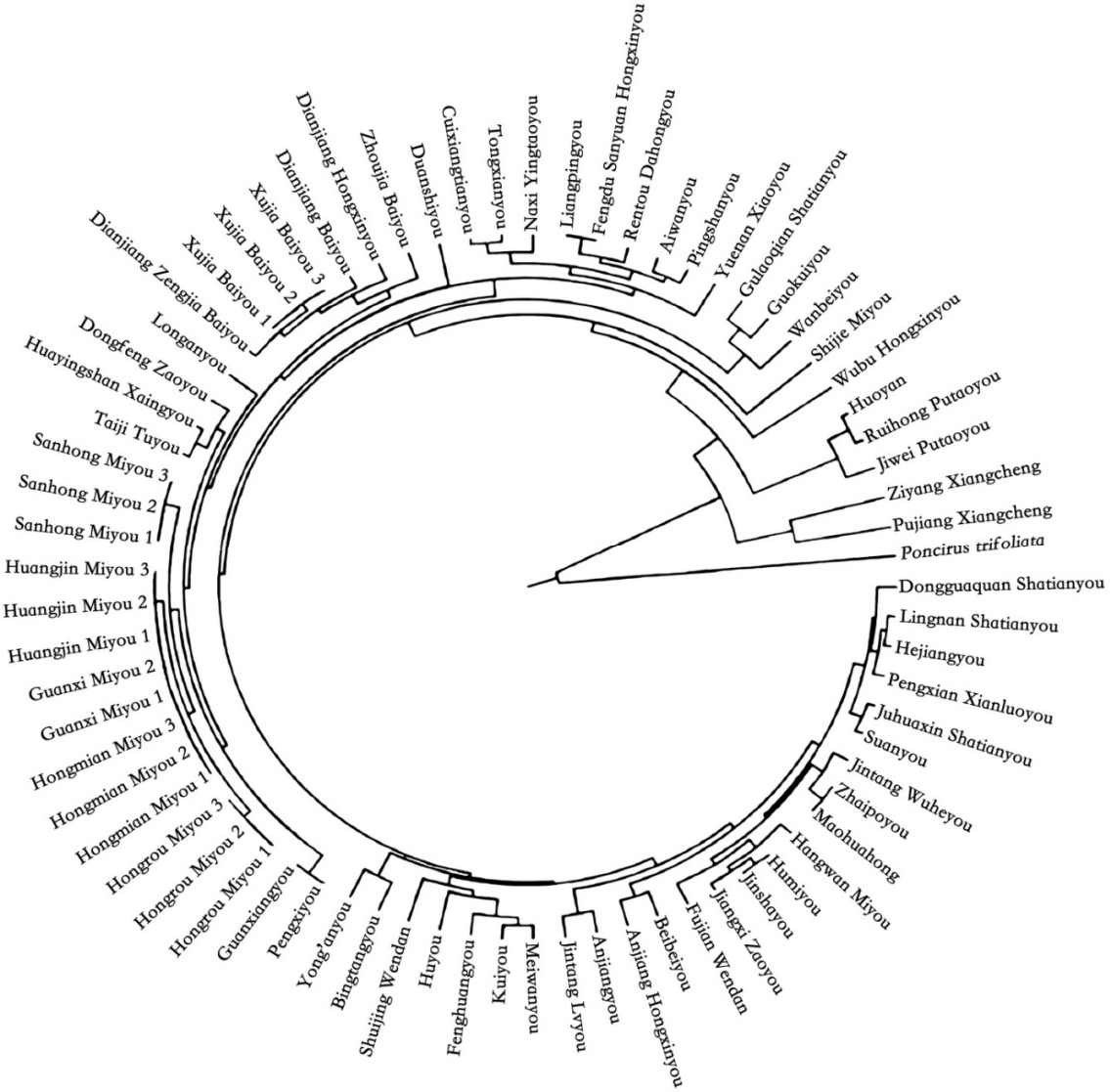

FIGURE 2. Clustering analysis of 73 pummelo germplasms and rootstocks based on SSR markers

DISCUSSION

SSR markers are effective tools to identify citrus germplasms [13]. Liu [14, 15] used 9 pairs of SSR primers to distinguish 122 pummelo germplasms and the relative species. Recently, Liu [16] indicted that there was a rich genetic diversity of pummelo germplasms resources in Zhejiang Province, and four SSR primers could be used for rapid and accurate identification of local pummelo germplasms. In this study, 15 pairs of SSR primers could differentiate pummelo germplasm and rootstocks in Sichuan Basin, which provided abundant genetic variation.

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 [17]. 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. [17] 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.

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