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Simple Sequence Repeat Molecular Marking Analysis of the Genetic Relationship between Zhaoqing Local Fine Citrus Breeds and Other Citrus and Allied Plants

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Abstract

The genetic relationship between Zhaoqing local citrus breeds and other citrus and allied plants was studied using the simple sequence repeat (SSR) molecular marker technique. Among 34 pairs of citrus SSR primers, this study selected 13 primer pairs with high polymorphism to expand 83 bands with 100% polymorphism positive rate. Through cluster analysis, this study effectively differentiated the species, breeds, and even strains of citrus, poncitrus, and atalantia correa. Thus, SSR molecular marker techniques can adapt to the classification of the status research of local citrus germplasm resources. A close genetic relationship was found among Zhaoqing local citrus breeds. This study provided evidence that *Citrus haniana* Hort. ex Tseng Sihuihanggan is the female parent of *C. nobilis* Lour. gonggan by combining morphological and other molecular biology research.

Keywords: local citrus breeds, simple sequence repeat, cluster analysis, genetic relationship, origin

1. Introduction

Guangdong Province is one of the major citrus-producing areas in China. Zhaoqing City is the no.1 producer in Guangdong, with the largest output and total plantation area. By the end of 2011, the plantation area of citrus in Zhaoqing reached 960 thousand mu (actual production area: 850 thousand mu) with a total output of 850 thousand tons. In this region, *Citrus flamea* Hort. ex Tseng shiyueju (Seedless) and *C. nobilis* Lour. gonggan are the main breeds planted in most farms. These breeds are characterized by their thin peel, crisp pulp, meltable residue, and sweet taste. Their superior qualities are well known all over the country (Ji, 2011).

Zhaoqing, Sihui County has a very long history of citrus plantation. The purposive plantation practice can be found in the historical records compiled during the Han Dynasty. According to “Sihui County Annals” (edited in the period of Emperor Guangxu during the Qing Dynasty), the citrus plants in Sihui earned their names ahead of the breeds planted in other areas. Its abundant citrus resources included more than 20 massively cultivated breeds (this figure may exceed 30 if all the strains were counted in). According to the “China Citrus Technology Book (complied in 1992 by the Citrus Research Institute of the Chinese Academy of Agricultural Sciences),” Sihui has more than 30 breeds of citrus, including more than 20 local breeds. Before the 1990s, *C. haniana* Hort. ex Tseng Sihuihanggan was the dominant breed planted in Sihui. Later in the 1990s, however, *C. flamea* Hort. ex Tseng wuyueju, *C. nobilis* Lour. gonggan, *Citrus flamea* Hort. ex Tseng bayueju and *Citrus flamea* Hort. ex Tseng shiyueju(Seedless)became more popular in this region because of their more superior qualities. Furthermore, the breed structure of the citrus in Sihui had been optimized through continuous technology improvement. After years of dedicated promotions, *C. nobilis* Lour. gonggan and *Citrus flamea* Hort. ex Tseng shiyueju (Seedless) are now the main breeds planted in Zhaoqing (Ji, 2011).

Citrus plants may easily hybridize, and open pollinations can also be easily carried out among them. Many new local breeds or breeds with mutated genes are generated through natural hybridization, artificial cultivation,

selection, and plantation because citrus plants can reproduce asexually, which makes their classification very complicated. The citrus breeds currently planted in Zhaoqing are mainly the local ones coming down historically whose parents and origin are not quite clear. No systematic research has been carried out on them and their breeding. For example, according to Sihui County Annals (edited in the period of Emperor Guangxu in the Qing Dynasty), *C. nobilis* Lour. gonggan originated locally from Sihui through the natural hybridization of orange and tangerine. Although such breeds have been biologically classified and named according to their morphological features, their classification status differs greatly because different scholars may have different systematic classification methods for citrus plants. For example, *C. nobilis* Lour. gonggan is sometimes named as *Sinocitrus suhuiensis*, Mandarin breed of Sinocitrus Tseng, or *Citrus nobilis* breed of Sinocitrus Tseng (Ji, 2011). Thus, some breeds may have different morphological characteristics but very close genetic relationships. The status classification of the citrus plants in Zhaoqing should be explicitly defined using modern classification technologies to establish a theoretical basis for further research. Simple sequence repeat (SSR) markers are widely used in scientific fields such as genetic diversity analysis, germplasm identification, genetic map construction, and gene mapping because of their advantages, including high polymorphism, co-dominance, reliability, and repeatability. A certain amount of SSR primers developed in the gene complexes of citrus plants was adopted by many scholars in genetic diversity studies of lemon, pomelo, and fortunella (Wang, 2010; Pang, 2003; Riaz, 2003; Maria, 2003; Kijias, 1997).

In recent years, the author of the present article has studied the citrus breeds in Zhaoqing by covering the following aspects: random amplified polymorphic DNA (RAPD) marking technique, chloroplast gene, isozyme, quality analysis, nutrition test, breeding of new breeds, history, culture, and so on (Ji, 2007, 2010, 2011, 2012). Furthermore, research results have established a certain theoretical basis for related studies on the breed identification, fine breed selection, genetic relationship, and classification of these local citrus breeds. In the research of *C. nobilis* Lour. gonggan's origin, consistencies in the molecular and morphological evidence about *C. nobilis* Lour. gonggan's classification status were studied using the RAPD molecular marker method. The possible female parent of *C. nobilis* Lour. gonggan–*C. haniana* Hort. ex Tseng Sihuihanggan was identified through RAPD and chloroplast tRNA-Leu(trnL) gene evidence. Consequently, a new breed of seedless *C. nobilis* Lour. gonggan was developed. Accordingly, this article aims to discuss the genetic relationship and origin of various citrus breeds and related plant species using the SSR marker method. SSR primers were selected to analyze local citrus, Sinocitrus, or other plants with very close genetic relationships. The results of the present study may provide solid supporting evidence for their classifications and breeding work.

2. Samples and Testing Methods

2.1 Samples Tested

Thirty test samples are prepared (Table 1), including Citrus (28 samples), Poncitrus (1 sample), and Atalantia (1 sample, used as out-group). Breeds 1–20 and 36 are collected from Pomology Research Institute, Guangdong Academy of Agricultural Sciences. Breeds 21–35 are collected from Pomology Research Institute, Zhaoqing College. Among these breeds, *C. flamea* Hort. ex Tseng Mashiju, *C. haniana* Hort. ex Tseng Sihuihanggan, *C. haniana* Hort. ex Tseng Sihuigan, *C. flamea* Hort. ex Tseng bayueju, *C. flamea* Hort. ex Tseng shiyueju(Big fruit shape), *C. flamea* Hort. ex Tseng shiyueju (Seedless), and *Citrus sinensis* osbeck Lanhuacheng No.4 are the citrus breeds present in the local plantation history. Currently, *C. nobilis* Lour. gonggan and *C. flamea* Hort. ex Tseng shiyueju(Seedless) are the dominant breeds planted in this region. The scientific names used in "Chinese Fruit Species: Citrus (Zhou, 2009)" are adopted for all the test materials.

2.2 Selection of SSR Primers

The primers described in References Riaz (2003), Maria-Teresa (2003), and Kijias (1997) are referred to, and 34 pairs of SSR primers are screened out (Table 2), which are synthesized by Shanghai Bioengineering Co., LTD. Breeds *C. grandis* (L.) Osbeck (hongroumiyou), *Poncirus trifoliata* (L.) Raf, *C. limon* (L.) Burm. f. Youlikeningmeng (Eureka Lemon), *C. sinensis* Osbeck Niuheerqicheng (Newhall Navel Orange), *C. nobilis* Lour. gonggan, and *Atalantia buxifolia* (Poir.) Oliv. are selected to screen out the SSR primers further. A total of 13 primer pairs with high polymorphism are selected from the 34 pairs of SSR for the tests. The codes of primer sequence with polymorphism are CTT01, TAA15, SS18, TAA1, SS16, CSSR036, CMS30, CAC19, CMS24, CMS14, CMS21, TAA3, CMS20.

The amplification product is tested in 1×TBE buffer solution using 1.5% agarose gel electrophoresis.

Table 1. The name of samples tested

NO.	*Latin or English name*
1	*Citrus grandis (L.)Osbeck(kekouyou)*
2	*Citrus tangerina Tanaka(jiangxihongju)*
3	*Citrus grandis (L.)Osbeck(hongroumiyou)*
4	*Poncirus trifoliata (L.) Raf*
5	*Citrus limon (L.) Burm.f.Youlikeningmeng(Eureka Lemon)*
6	*Citrus sinensis Osbeck Niuheerqicheng(Newhall Navel Orange)*
7	*Gongneiyiyuan(Miyauchi Iyokan)*
8	*Citrus reticulata Blanco xinshengxi NO.3penggan*
9	*Moketeju (Murcutt tangerine)*
10	*Citrus nobilis Lour.Nangan No.20*
11	*Qiuhuijuyou(Fallglo Tangelo)*
12	*Citrus reticulata Blanco No.830*
13	*Nowajuyou(Nova tangelo)*
14	*Citrus sinensis Osbeck Qingjiaqicheng(Seike Navel orange)*
15	*Citrus sinensis Osbeck Fulingxiacheng (Valencia Orange)*
16	*Citrus nobilis Lour. Xingjinwenzhoumigan(okitsu wase)*
17	*Citrus sinensis osbeck tangcheng*
18	*Citrus sinensis osbeck hongjiangcheng*
19	*Citrus grandis(L.)osbeck shatianyou*
20	*Citrus haniana Hort. ex Tseng Nianju*
21	*Citrus junons Sieb .ex . Tanaka*
22	*Citrus flamea Hort. ex Tseng Mashiju*
23	*Citrus haniana Hort. ex Tseng Sihuihanggan*
24	*Citrus haniana Hort. ex Tseng Sihuigan*
25	*Citrus nobilis Lour. gonggan*
26	*Citrus flamea Hort. ex Tseng bayueju*
27	*Citrus flamea Hort. ex Tseng shiyueju(Big fruit shape)*
28	*Citrus flamea Hort. ex Tseng shiyueju(Seedless)*
29	*Citrus sinensis osbeck Lanhuacheng No.4*
30	*Atalantia buxifolia (Poir.) Oliv.*

2.3 SSR Test and Its Amplification Products

Modified CTAB (hexadecyl trimethyl ammonium bromide) method (Ji, 2011; Clark, 1998) is used to abstract the genomes and total DNA. The purity and density of the DNA are determined using 1% agarose gel electrophoresis and micro-spectrophotometer. The DNA sample (100 ng) is collected as the PCR template according to the DNA density.

The PCR mix serves as the Hotstart system. The PCR reaction system includes:

- DNA 100 ng.
- $MgCl_2$ 2.75mmol·L^{-1}.
- upstream primer/downstream primer 0.2 μmol L^{-1} separately.
- dNTP 0.2 μmol·L^{-1}.
- Taq polymerase 0.1 U.

The PCR reaction procedures are as follows:

- Pre-denaturation at 94 °C for 5 min.
- Denaturation at 94 °C for 1 min.
- Annealing at 53 °C for 1 min.
- Stretching at 72 °C for 1 min.
- 35 circles.
- Stretching at 72 °C for 10 min after the circulation.
- Storage at 4 °C.

2.4 Band Analysis

The numbers of the DNA amplification and polymorphism bands of the tested samples are recorded according to the existence of the SSR amplification bands to build up 1.0-type database. Cluster analysis is performed using UPGMA**,** and a cluster tree is built using the NTSYSpc2.1 analysis software. Meanwhile, the genetic similarity coefficient is calculated.

Table 2. SSR primer pairs of citrus for this paper study

No	primer	Forward primers(5'→ 3') Reverse primers(5'→ 3')
1	TAA1	GACAACATCAACAACAGCAAGAGC
		AAGAAGAAGAGCCCCCATTAGC
2	CMS46	TCAAACATCAGACGAAGCAA
		TGAATCTTTTGCCGAATTTTG
3	CMS31	CGTGCAGAGAAACTCAGATCC
		GCTGAAAAAGATTCATTTTGCC
4	CMS8	CCAAACATCTGCGGATCC
		AGAAGAACCCAGATTCCAAATG
5	CMS47	GGATCCTCCACCATCTCGTA
		TTCTTCTTCCATGCCGACTT
6	CMS45	CGACCACTCCACCTACGATG
		GCCGTTAATTCCTGCTTTCA
7	CSSR038	GCTCCTCGAATGAGAATGAAATGA
		TGGTTGTGCGAAAATGAAGAGATA
8	CSSR036	AAAAATCGAAATCGAGCACCC
		GAAGTAACGGAGAATTCCGATGAG
9	CSSR050	TTCACCACAAACGAAGACTCAGAC
		CTGTAATCCACTCGGTAATCCGAC
10	CSSR052	CGAAGAAGAATTGAAAGAGCCAGA
		CAACAGATTTGTTACTGGAAGGGG
11	SS17	TTCATTTGGAACAAAACCCAATTC3
		GCTGCTAATCACAGCATCAAGAGA
12	SS16	AGTGAACTGTCCATTGGATTTTCG
		GTGTTGAATCCCGACCTTCTACC
13	CSSR051	TAGGTTCTCTTTCAACCCCCTTTC
		CTGCTTCGGCTGTAATTGTGATT
14	SS18	CCTCAGCTCTAGCAAAAGCACATT
		AGAGGCTATAGATCGTGGATGCAG
15	SS2	TTTATTCACCGCTCAAGGACT

		TTAGGGGTGGAAAACATGGA
16	CSSR020	ACATTCGCATTCTCCACT
		TTTGTCTCATCACCTTCG
17	SS15	GCTTTCGATCCCTCCACATA
		GATCCCTACAATCCTTGGTCC
18	CSSR015	ATACGATGCGTGAACTGC
		TACCTTTCTTTCTCCTCTGT
19	AG14	AAAGGGAAAGCCCTAATCTCA
		CTTCCTCTTGCGGAGTGTTC
20	CAT01	GCTTTCGATCCCTCCACATA
		GATCCCTACAATCCTTGGTCC
21	CCT01	TCAACACCTCGAACAGAAGG
		CCCACATGCTAGCACAAAGA
22	TAA52	GATCTTGACTGAACTTAAAG
		ATGTATTGTGTTGATAACG
23	cAGG9	AATGCTGAAGATAATCCGCG
		TGCCTTGCTCTCCACTCC
24	CAC19	ACAACCTTCAACAAAACCTAGG
		AAGACTTGGTGCGACAGG
25	CMS30	AACACCCCTTGGAGGGAG
		GCTGTTCACACACACAACCC
26	CMS24	TTATTGTCCCCAATTGTGAGC
		TCCAGATTGAGGGGAAAAAG
27	CMS21	TAGGCCAAATCTTATTCATGCC
		TCAGGGTCATAAGGAATGGC
28	CMS14	TGGCTTCTCTTCTACTAGAACGG
		ACGCCACGTAAGCAATAACC
29	CMS7	CAGGATGCTTGTTGGTGATG
		ACAGTGGATACAAACATGCTGC
30	CAC15	TAAATCTCCACTCTGCAAAAGC
		GATAGGAAGCGTCGTAGACCC
31	CMS10	TATGGTGGCAGATTAACAGCC
		TACTCCGGAGAAAGATTTGGG
32	CMS20	GGAGCATATAAGCATAAACACC
		AGGAAAACGCATAAACCGTG
33	TAA3	AGAGAAGAAACATTTGCGGAGC
		GAGATGGGACTTGGTTCATCACG
34	TAA45	GCACCTTTTATACCTGACTCGG
		TTCAGCATTTGAGTTGGTTACG

3. Result Analysis

3.1 The DNA's SSR Amplification Results of the Tested Samples

All 13 primer pairs are amplified with an appearance of clear polymorphism bands (Figure 1). The amplification band number differs from primer to primer, ranging from 2 to 16 (polymorphism band number: minimum of 2 and maximum of 16). Among all the selected primers, CAC19, CMS30, TAA1, TAA15, and CTT01 have more

amplification and polymorphism bands than the others. A total of 83 bands containing 83 polymorphism bands are amplified. Each primer has an average of 6.4 polymorphism bands. The average polymorphism is 100%. Regarding the local citrus breeds in Zhaoqing, 36, 33, 36, 28, 29, 34, 30, and 29 bands are amplified for *C. flamea* Hort. ex Tseng Mashiju, *C. haniana* Hort. ex Tseng Sihuihanggan, *C. haniana* Hort. ex Tseng Sihuigan, *C. nobilis* Lour. gonggan, *C. flamea* Hort. ex Tseng bayueju, *C. flamea* Hort. ex Tseng shiyueju (Big fruit shape), *C. flamea* Hort. ex Tseng shiyueju (Seedless), and *C. sinensis* osbeck Lanhuacheng No.4, respectively.

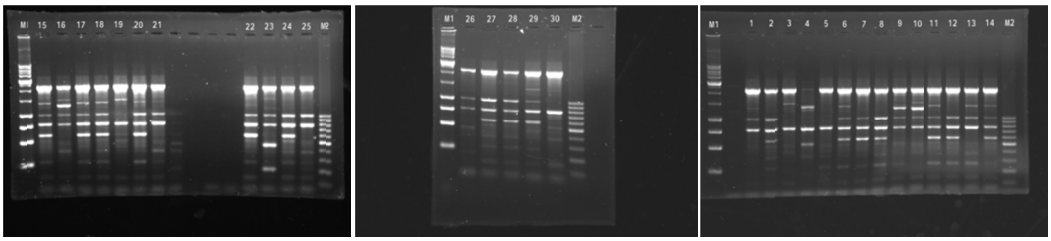

Figure 1. Amplification results of 30 materials with CTT01 primer

Note: M1: 1 Kb Marker; M2: 100 bp Marker; Nos. 1 to 30 are the test materials, see Table 1.

3.2 Cluster Analysis of the Tested Samples

Cluster analysis of the 1.0 data of all the bands is performed using UPGMA to build a cluster tree (Figure 2).

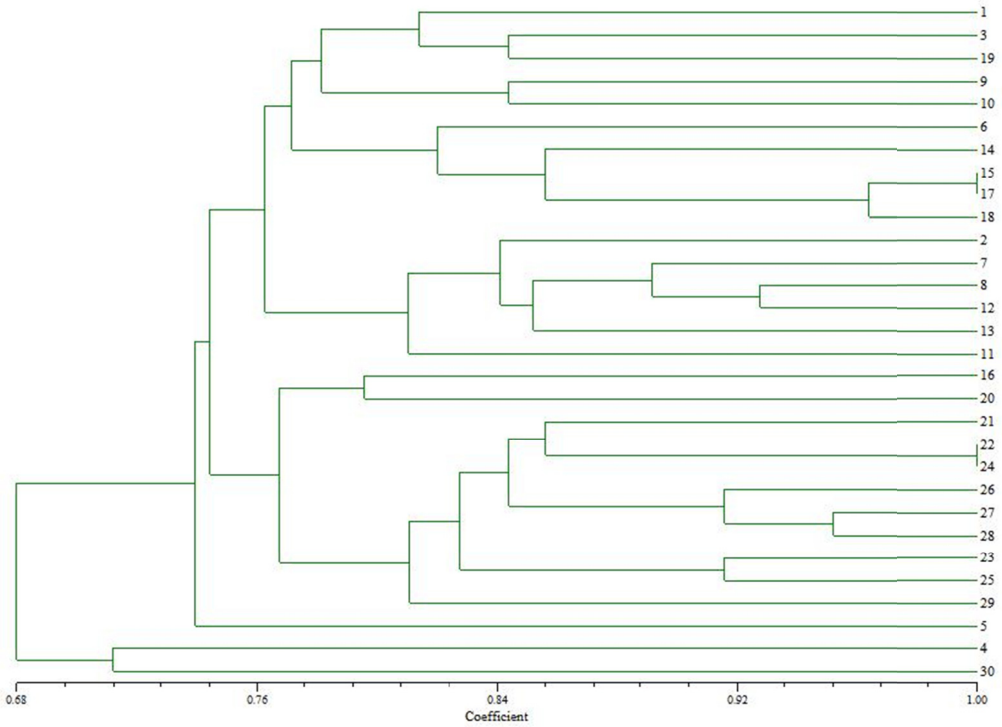

Figure 2. Dendrogram for the 30 materials derived from cluster analysis of SSR data

Nos. 1 to 30 are the test materials, see Table 1.

All the tested samples can be accurately classified as citrus, poncitrus, or atalantia using genetic similarity coefficient 0.7 as the criteria (Table 3). The 28 samples classified as citrus using genetic similarity coefficient 0.76 as the criteria can be further divided into six groups:

Group 1: *C. grandis* (L.) Osbeck (kekouyou), *C. grandis* (L.) Osbeck (hongroumiyou), *C. grandis* (L.) osbeck shatianyou.

Group 2: Moketeju (Murcutt tangerine), *C. nobilis* Lour.Nangan No. 20.

Group 3: Gongneiyiyuan (Miyauchi Iyokan), *C. sinensis* Osbeck Qingjiaqicheng (Seike Navel orange), *C. sinensis* Osbeck Fulingxiacheng (Valencia Orange), *C. sinensis* osbeck tangcheng, *C. sinensis* osbeck hongjiangcheng.

Group 4: *C. tangerina* Tanaka (jiangxihongju), Gongneiyiyuan (Miyauchi Iyokan), *C. reticulata* Blanco xinshengxi NO. 3 penggan, Qiuhuijuyou (Fallglo Tangelo), *C. reticulata* Blanco No.830, Nowajuyou (Nova tangelo).

Group 5: *C. nobilis* Lour. Xingjinwenzhoumigan (okitsu wase), *C. haniana* Hort. ex Tseng Nianju, C. *junons* Sieb .ex . Tanaka, *C. flamea* Hort. ex Tseng Mashiju, *C. haniana* Hort. ex Tseng Sihuihanggan, *C. haniana* Hort. ex Tseng Sihuigan, *C. nobilis* Lour. gonggan, *C. flamea* Hort. ex Tseng bayueju, *C. flamea* Hort. ex Tseng shiyueju (Big fruit shape), *C. flamea* Hort. ex Tseng shiyueju (Seedless), *C. sinensis* osbeck Lanhuacheng No. 4.

Group 6: *C. limon* (L.) Burm.f.Youlikeningmeng (Eureka Lemon).

Mandarin, tangerine, orange, lemon, and pomelo can then be differentiated from one another. These results comparatively match with traditional botanical classifications. In Group 4, *C. reticulata* Blanco xinshengxi NO. 3 penggan and *C. reticulata* Blanco No. 830 are clustered into one category (0.93). According to the genetic similarity coefficient (Table 3), all the other citrus breeds (except *C. nobilis* Lour. Xingjinwenzhoumigan (okitsu wase) and C. *junons* Sieb .ex. Tanaka in Group 5) are the unique ones from Guangdong Province with very close genetic relationships in Group 5. Among these breeds, *C. haniana* Hort. ex Tseng Sihuihanggan and *C. nobilis* Lour. gonggan (0.92), *C. flamea* Hort. ex Tseng Mashiju and *C. haniana* Hort. ex Tseng Sihuigan (1.00), and *C. flamea* Hort. ex Tseng shiyueju (Big fruit shape) and *C. flamea* Hort. ex Tseng shiyueju (Seedless) (0.95) are separately clustered into one category.

Table 3. Coefficient of genetic similarity of 30 materials

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1.00																													
0.77	1.00																												
0.82	0.76	1.00																											
0.69	0.72	0.72	1.00																										
0.70	0.73	0.78	0.67	1.00																									
0.78	0.75	0.72	0.61	0.67	1.00																								
0.77	0.83	0.73	0.63	0.71	0.80	1.00																							
0.75	0.86	0.71	0.67	0.64	0.80	0.90	1.00																						
0.80	0.76	0.78	0.75	0.78	0.77	0.76	0.78	1.00																					
0.78	0.77	0.77	0.73	0.75	0.76	0.77	0.77	0.84	1.00																				
0.75	0.78	0.76	0.72	0.69	0.70	0.76	0.83	0.76	0.77	1.00																			
0.77	0.86	0.73	0.72	0.71	0.77	0.88	0.93	0.78	0.80	0.86	1.00																		
0.78	0.82	0.77	0.71	0.75	0.76	0.84	0.84	0.82	0.78	0.82	0.87	1.00																	
0.80	0.81	0.73	0.72	0.73	0.82	0.76	0.76	0.81	0.82	0.76	0.81	0.80	1.00																
0.81	0.77	0.77	0.66	0.77	0.83	0.77	0.72	0.77	0.73	0.72	0.80	0.78	0.87	1.00															
0.78	0.70	0.77	0.71	0.77	0.66	0.72	0.65	0.75	0.83	0.70	0.72	0.73	0.72	0.78	1.00														
0.81	0.77	0.77	0.66	0.77	0.83	0.77	0.72	0.77	0.73	0.72	0.80	0.78	0.87	1.00	0.78	1.00													
0.82	0.78	0.78	0.65	0.78	0.80	0.78	0.71	0.73	0.72	0.73	0.78	0.77	0.83	0.96	0.80	0.96	1.00												
0.81	0.75	0.84	0.71	0.80	0.69	0.72	0.70	0.80	0.76	0.72	0.70	0.76	0.75	0.81	0.81	0.81	0.80	1.00											
0.80	0.78	0.71	0.65	0.69	0.70	0.78	0.76	0.71	0.70	0.76	0.78	0.72	0.71	0.82	0.80	0.82	0.83	0.80	1.00										
0.78	0.75	0.70	0.71	0.77	0.66	0.75	0.70	0.77	0.81	0.72	0.75	0.76	0.77	0.76	0.83	0.76	0.77	0.83	0.80	1.00									
0.71	0.75	0.63	0.66	0.70	0.64	0.75	0.72	0.77	0.71	0.72	0.77	0.76	0.75	0.76	0.76	0.76	0.75	0.78	0.82	0.86	1.00								
0.77	0.81	0.69	0.70	0.73	0.67	0.76	0.73	0.76	0.75	0.69	0.78	0.77	0.78	0.77	0.70	0.77	0.78	0.82	0.78	0.82	0.82	1.00							
0.71	0.75	0.63	0.66	0.70	0.64	0.75	0.72	0.77	0.71	0.72	0.77	0.76	0.75	0.76	0.76	0.76	0.75	0.78	0.82	0.86	1.00	0.82	1.00						
0.76	0.82	0.72	0.69	0.77	0.66	0.77	0.75	0.75	0.73	0.70	0.77	0.73	0.77	0.78	0.73	0.78	0.80	0.86	0.80	0.83	0.83	0.92	0.83	1.00					
0.70	0.76	0.69	0.70	0.78	0.63	0.69	0.69	0.73	0.75	0.69	0.73	0.75	0.73	0.70	0.75	0.70	0.71	0.80	0.73	0.84	0.84	0.83	0.84	0.87	1.00				
0.71	0.75	0.67	0.71	0.75	0.66	0.70	0.72	0.77	0.76	0.72	0.77	0.73	0.75	0.73	0.73	0.73	0.72	0.81	0.77	0.83	0.83	0.82	0.83	0.81	0.89	1.00			
0.71	0.77	0.67	0.73	0.75	0.64	0.70	0.72	0.75	0.76	0.72	0.77	0.73	0.77	0.73	0.76	0.73	0.72	0.81	0.77	0.86	0.86	0.82	0.86	0.83	0.94	0.95	1.00		
0.70	0.78	0.73	0.75	0.76	0.67	0.73	0.71	0.81	0.77	0.69	0.76	0.75	0.83	0.80	0.77	0.80	0.76	0.80	0.71	0.82	0.80	0.76	0.80	0.80	0.83	0.82	0.87	1.00	
0.69	0.67	0.65	0.71	0.67	0.61	0.63	0.65	0.70	0.66	0.67	0.67	0.66	0.67	0.64	0.61	0.64	0.60	0.73	0.60	0.69	0.64	0.67	0.64	0.69	0.67	0.71	0.69	0.72	1.00

Note: Nos. 1 to 30 are the test materials, see table 1.

4. Discussions

The SSR amplification results show that the positive rate of polymorphism is 100%. The amplification bands generated by different samples tested with different primers vary greatly. The genetic similarity coefficient is between 0.60 and 1.00, which shows a wide genetic backgroup of the selected test samples. A total of 83 amplification bands are found. Each pair of primer has an average of 6.4 bands, showing a comparatively high richness. The clustering results show that the SSR molecular marker technique can reveal genetic diversity and

differentiate citrus, poncitrus, and atalantia from one another. This technique can also further divide citrus into different groups, including mandarin, tangerine, orange, lemon, and pomelo. The results are basically in accordance with traditional botanical classifications. Two different strains (*C. reticulata* Blanco No. 830 and *Citrus reticulata* Blanco xinshengxi NO. 3 penggan) of *Citrus reticulata* Blanco are clustered into one category (0.93) and are used as test samples. These two strains can be clearly differentiated from each other. Meanwhile, the different bud mutation strains (*C. flamea* Hort. ex Tseng shiyueju (Big fruit shape) and *C. flamea* Hort. ex Tseng shiyueju (Seedless)) of *C. flamea* Hort. ex Tseng shiyueju are also used as test samples. These strains are also clustered into one category (0.95) and can be clearly differentiated from each other. This method is proven to be effective in identifying citrus species, breeds, and strains.

The citrus breeds planted in Zhaoqing mainly originated from Sihui. According to the subspecies classification system proposed by Tanaka (1969, 1977), the citrus plants originated from Sihui are categorized into one species (species in classification) named as "*C. suhuiensis* Hort. ex Tanaka." This species is also pointed out to be distributed in Guangdong Sihui to Guangzhou and Hainan Island, China. Zeng (1960) also classified citrus plants from Sihui into a species called *C. suhuiensis* (Tanaka), including *C. haniana* Hort. ex Tseng Sihuihanggan, *C. haniana* Hort. ex Tseng Sihuigan, and *C. nobilis* Lour. gonggan. These results imply the important position of the different citrus breeds in Sihui. According to the results of genetic similarity analysis using the SSR molecular marker technique, various citrus breeds planted in Sihui have a comparatively close genetic relationship and can be classified into one category with *C. nobilis* Lour xingjinwenzhoumigan (okitsu wase). However, this category also includes oranges C. *junons* Sieb .ex. Tanaka and *C. sinensis* osbeck Lanhuacheng No.4 (Rootstock). Therefore, the genetic relationship between *C. sinensis* osbeck Lanhuacheng No. 4, which originated from Sihui, and other local citrus plants should be studied in the future.

According to past studies carried out by the author of this article, Zhou (2009)'s viewpoint is supported by the PAPD molecular marker technique. In addition, *C. nobilis* Lour. gonggan is confirmed to be a breed of Sinocitrus Tseng Macroacrumen *C. nobilis*. *C. nobilis* jiaogan and *C. nobilis* wenzhoumigan are also classified as *C. nobilis*. Studies on the chloroplast gene *tmL* speculated that *C. nobilis* Lour. gonggan is a natural hybrid species, with *C. haniana* Hort. ex Tseng Sihuihanggan being its female parent. In this article, *C. haniana* Hort. ex Tseng Sihuihanggan and *C. nobilis* Lour. gonggan are classified into one category, and *C. haniana* Hort. ex Tseng Sihuihanggan is ascertained to be the female parent of *C. nobilis* Lour. gonggan by *tmL* and SSR evidence.

Sihui has been a very important trade center from ancient times to the present. Consequently, it serves as the main hub for citrus breeds to be introduced from one place to another. Countless citrus breeds introduced from other places, together with the locally originated ones, have formed a huge resource pool of citrus genes. Different breeds have been generated in different periods through natural hybridization, bud mutation, and so on. However, most of the new breeds generated (including *C. nobilis* Lour. gonggan and *C. flamea* Hort. ex Tseng shiyueju (Seedless)) are only traditional farm types without any modern methods of breeding and cultivation. Thus, many citrus trees produced through the reproduction method of air layering still exist in some old orchards. These casual operations of breeding and plantations are considered as one of the main reasons why some citrus gene resources in Sihui have been lost forever. In the 1990s, *C. haniana* Hort. ex Tseng Sihuihanggan was a planted breed. This breed was replaced with some other breeds because it cannot cater for the taste of modern people, causing it to be on the verge of extinction. For example, *C. nobilis* Lour. gonggan and *C. flamea* Hort. ex Tseng shiyueju (Seedless) are currently planted breeds with different shapes. However, the analysis results of PAPD, *trnL*, and SSR suggest that their very close genetic relationships are worthy of further studies. Furthermore, they have shown great differences in the resistance to citrus vein phloem degeneration as well as to anthracnose. Thus, each of them has special advantages. The abundant citrus gene resources in Sihui County can be used not only as excellent materials for breeding new breeds, but also as suitable resources for related genetic studies (Ji, 2011).

5. Conclusions

(1) The polymorphism positive rate of SSR molecular marking is high, with over 100% polymorphic bands. The cluster analysis UPGMA in polymorphic bands can effectively differentiate the species, breeds, and even strains of citrus, poncitrus, and atalantia correa. Thus, using SSR molecular marker techniques can adapt to the classification of the status research of Zhaoqing local citrus germplasm resources.

(2) The cluster analysis result shows that a close genetic relationship exists among Zhaoqing local citrus breeds. Historical background research suggests that these citrus species have different species because natural hybridization leads to the exchange of genetic materials or because the reservation of bud mutation leads to the formation of different species with similar genetic background.

(3) The parent of citrus species with an unidentified parental origin cannot be identified using only one marking technique. Morphological and molecular identification techniques should be combined to provide sufficient evidence. Furthermore, the results of PAPD, SSR, chloroplast gene *trnL*, and morphology show that the female parent of *C. nobilis* Lour. gonggan is *C. haniana* Hort. ex Tseng Sihuihanggan.

(4) Being an important citrus natural resource library of Guangdong Province, the citrus plants of Zhaoqing Sihui are worthy of germplasm resource collection, preservation, research and redevelopment, and utilization.

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