African Journal of Biotechnology Vol. 10(64), pp. 13982-13990, 19 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1501
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Using optimized random amplified polymorphic DNA (RAPD) markers to identify the category status of *Citrus nobilis* Lour. Gonggan

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Accepted 23 September, 2011

***Citrus nobilis* Lour. Gonggan is an excellent fruit variety which is widely planted in South China. The origin of Gonggan is not clear. It is conjectured that its origin is from a cross between tangerine and orange; however, there is no direct evidence to confirm this. Here, we applied the optimized random amplified polymorphic DNA (RAPD)-PCR to amplify genus *Citrus* species: 1) to better understand the genetic relationship between *C. nobilis* Lour. Gonggan and other *Citrus* species; and (2) to address the phylogenetic relationship among Citrus species. A total of 21 RAPD primers were used to screen 4 *Citrus* species and 10 of them efficiently amplified the genomic DNA of 23 *Citrus* accessions. A total of 87 locus/alleles were generated by those 10 primers with an average of 97.7% polymorphic. Our data supported that *C. nobilis* Lour. Gonggan belongs to a big group with most tested tangerine and orange and a subgroup with *Citrus haniana* and *Citrus flamea*, implying that either *C. haniana* or *C. flamea* is likely to be one of the parents of *C. nobilis* Lour. Gonggan.**

Key words: *Citrus nobilis* Lour. Gonggan, random amplified polymorphism DNA (RAPD), phylogenetic relationship.

INTRODUCTION

Citrus nobilis Lour. Gonggan is ranked the second widely planted cultivar in South China which has a most popular favorite golden thin peel and a honey taste and is awarded "Chinese famous fruit" by the China Fruit Marketing Association (Ji et al., 2007, 2009). In the Ming and Qing Dynasties, it was chosen as a tribute fruit to the imperial family. Sihui County is the origin of Gonggan in China. Based on the Sihui county records, Gonggan is the natural hybrid of tangerine and orange, but its exact parents is not recorded and as a variety it retained after several generation's selection. The local citrus varieties also include *C. flamea* Hort. ex Tseng shiyueju, *C. nobilis* Lour. Gonggan, and auxiliary cultivars of *C. flamea* Hort. ex Tseng bayueju and *C. flamea* Hort. ex Tseng wuyueju (Zhou and Ye, 2009). In addition, Sihui region has rich citrus resources in history, more than 20 cultivars or more than 30 strains were planted in Sihui region. Gonggan most likely has some relationship with some of the historical planted cultivars. Random amplified polymorphic DNA (RAPD) is a simple and fast DNA molecular marker technique to randomly amplify DNA fragments under low-stringency conditions by short t oligonucleotides (Williams et al., 1990). RAPD has been widely used to identify mutation, genetic diversity, mapping and molecular assistant selection (Liu and Hu, 1998; Pan, 2002). Here, RAPD technique was applied to identify the origin and phylogenetic relationship of Gonggan.

Twenty three *Citrus* cultivars (2 accessions) and RAPD-PCR reactions were conducted. Our results show the phylogenic relationship of Citrus cultivars and potential patents of *C. nobilis* Lour. Gonggan's.

MATERIALS AND METHODS

Plant materials

A total of 24 *Citrus* accessions' (Table 1) leaves were sampled and used. Species No.1 to 17 and 19 to 20 were provided by Fruit Tree

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Table 1. The *Citrus* accessions.

No.	Species	Belong to
1	*Citrus grandis* (L.) Osbeck.	Pomelo.
2	*Citrus tangerina* Tanaka.	Tangerine.
3	*Citrus grandis* (L.) Osbeck.	Pomelo.
4	*Poncirus trifoliata* (L.) Raf.	*Poncirus Raf.*
5	*Citrus limon* (L.) Burm. f. Youlikeningmeng (Eureka Lemon).	Lemon.
6	*Citrus sinensis* Osbeck Niuheerqicheng (Newhall Navel Orange).	Orange.
7	Gongneiyiyuan (Miyauchi Iyokan).	Mandarin.
8	*Citrus reticulata* Blanco xinshengxi NO.3 penggan.	Mandarin.
9	Moketeju (Murcutt tangerine).	Tangerine.
10	Qiuhuijuyou (Fallglo Tangelo).	Tangelo.
11	*Citrus reticulata* Blanco.	Mandarin.
12	Nowajuyou (Nova tangelo).	Tangelo.
13	*Citrus sinensis* Osbeck Qingjiaqicheng (Seike Navel orange).	Orange.
14	*Citrus sinensis* Osbeck Fulingxiacheng (Valencia Orange).	Orange.
15	*Citrus nobilis* Lour. Xingjinwenzhoumigan (okitsu wase).	Mandarin.
16	*Citrus sinensis* osbeck tangcheng	Orange.
17	*Citrus sinensis* osbeck hongjiangcheng.	Orange.
18	*Citrus grandis* (L.) osbeck shatianyou.	Pomelo.
19	*Citrus haniana* Hort. ex Tseng Nianju.	Tangerine.
20	*Citrus junons* Sieb. ex. Tanaka.	Mandarin.
21	*Citrus flamea* Hort . ex Tseng shiyueju.	Tangerine.
22	*Citrus nobilis* Lour. Gonggan.	Mandarin.
23	*Citrus flamea* Hort. ex Tseng bayueju.	Tangerine.
24	*Citrus flamea* Hort. ex Tseng 'wuyueju'.	Tangerine.

Research Institute, Guangdong Academy of Agricultural Sciences, China. No.18 and 21 to 24 were obtained from Fruit Tree Research Institute, Zhaoqing University, China.

Total genomic DNA extraction

Total genomic DNA was isolated based on modified method of Chen et al. (1997), Xiao (1995) and Dellaporta et al. (1983). 400 mg leaf samples were grinded in liquid nitrogen and added to 10 ml, preheated at 65℃ 1 × CTAB buffer (2% CTAB (W/V), 100 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA pH 8.0, 1.4 mol·L-1 NaCl) (with proper β-ME and PVP), incubated at 65℃ for 90 min, and then added equal volume chloroform/isoamyl alcohol solution and mixed well, it stayed in room temperature (RT) for 10 min, then centrifuged at 10 min of 4℃ at 10000 rpm/min; then the samples transferred the supernant to a fresh microtube, 1/10 volume 3M NaAc and 1 volume isopropyl alcohol were added, incubated at -20℃ for 30 min, then centrifuged at 10 min at 10000 r/min; the pellet was washed twice with 2 ml ice-cold 75% ethyl alcohol, dried in the air and re-suspended in 600 ul buffer, 3 ul RNaseA (final concentration 50 $ug \cdot ml^{-1}$) was added, kept at 37℃ for 30 min, equal volume of chloroform/isoamyl alcohol (pre-cold) solution to extract was added to the sample 1 to 3 times, then centrifuged at 10 min 4℃ at 10000 rpm/min; the supernant was transferred to a fresh micro tube and 5 $mol \cdot L^{-1}$ of NaCl was added to a final concentration among 0.1 to 0.14 $mol \cdot L^{-1}$, 2 volume of ice-cold ethyl alcohol was added, kept at 4℃ for 20 min, then centrifuged at 5 mins at 4℃ at 10000 rpm/min; the pellet was washed 2 to 3 times with ice-cold of 75% ethyl alcohol, dried in the air and re-suspension in TE buffer (PH = 7.4), then frozen at - 20℃ until it was used.

Detection of the DNA samples and optimization of the RAPD-PCR reaction condition

The DNA samples were run on a 1.0% agarose gel in 1 × TBE buffer with voltage of 5 V/cm for 60 min. DNA concentration was determined with the absorbance in 260 nm using spectrophotometer UV 1601 (Shimadzu Inc., Japan). The DNA concentration ($\mu g \cdot \mu l^{-1}$) = A260 × 50 × dilution factor/1000. In order to obtain the best PCR reaction condition, we keep all the components consistent except one component varied. The PCR products were separated on a 1.5% agarose gel in 1 × TBE buffer with voltage of 5 V/cm. The gel image was analyzed by Image Master VL system.

Data analysis

RAPD results were statistically analyzed. All the scorable bands were considered as single locus/allele. The loci were scored as present or absent. Bi-variate 1-0 data matrix was generated. Genetic distances were calculated using UPGMA procedure (Nei and Li, 1979).

RESULTS

Analysis of purified genomic DNA

Total genomic DNA was separated on agarose gel (Figure1); DNA bands about 30 kb were visible. DNA band appeared as sharp band, no smear indicating that

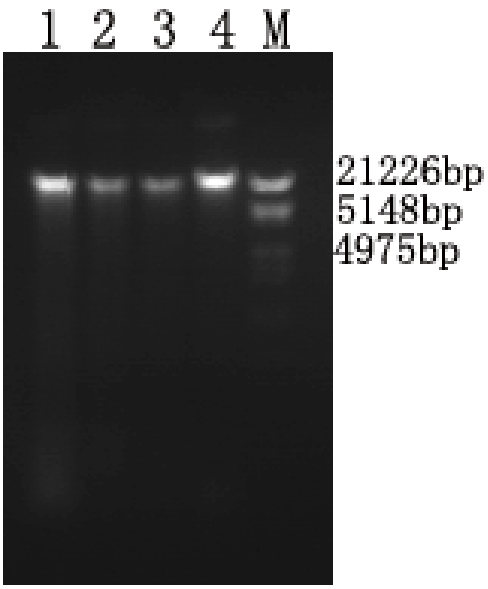

Figure 1. Electrophoresis analysis of the genomic DNA samples from leaves of four *Citrus* varieties. M: λDNA/EcoR*I*+Hind*III;* Lane 1: *C. flamea* Hort. ex Tseng shiyueju; lane 2: *C. flamea* Hort. ex Tseng bayueju; lane 3: *C. flamea* Hort. ex Tseng wuyueju; and lane 4: *C. nobilis* Lour. Gonggan.

samples are not contaminated with protein, RNA and polysaccharide. These genomic DNA were used for the RAPD analysis.

Optimize RAPD-PCR reaction

A total of 21 RAPD primers (Table 2) were selected for PCR analysis. The optimized RAPD-PCR reaction were conducted as follows (Liu and Hu, 1998; Liu et al., 2006; Fan et al., 2002): 10 ng template DNA 0.2 μmol·L^{-1} primer, 0.2 mmol·L^{-1} dNTPs, 0.1 U·$μl^{-1}$Taq, 2.75 mmol·L^{-1} Mg^{2+} and the PCR was performed as follows: 94℃ for 3 min; 94℃ for 1 min, 44℃ for 90 s, 73℃ for 2 min and repeated for 36 cycles; then 72℃ for 10 min.

Analysis of RAPD results

Initially, 21 primers were selected to conduct RAPD-PCR by using genomic DNA of *C. flamea* Hort. ex Tseng shiyueju (Sugar tangerines) (Figure 2A), *C. flamea* Hort. ex Tseng bayueju (August tangerines) (Figure 2B), *C. flamea* Hort. ex Tseng wuyueju (May tangerines) (Figure 2C) and *C. nobilis* Lour. gonggan (Figure 2D). Primers used in this experiment were listed (Table 2). Each primer generated 1 to 6 bands. 18 primers could amplify bands from *C. flamea* Hort. ex Tseng shiyueju, while only primer S10, S237, S266, S147 and S90 could generate clear and high polymorphic bands (Figure 2A). 12 primers could amplify bands from *C. flamea* Hort. ex Tseng 'bayueju', however, only S230, S253 and S418 could generate clear and high polymorphic bands (Figure 2B). 14 primers could amplify bands from *C. flamea* Hort. ex Tseng 'wuyueju', but only primer S266, S253, S99, S90, S227 and S418 were clear and high polymorphic bands (Figure 2C). 17 primers could generate bands from *C. nobilis* Lour. gonggan, 10 primers S418, S64, S71, S253, S147, S227, S238, S266, S28 and S8 were clear and high polymorphic bands (Figure 2D). Based on these results, 10 RAPD primers (S418, S64, S71, S253, S147, S227, S238, S266, S28 and S8) with the best amplification results in *C. nobilis* Lour. Gonggan were selected to further amplify all the *Citrus* accessions tested.

The result shows that each primer could amplify 3 to 16 bands. The percentage of polymorphic bands was from 92 to 100% (Table 3). Among these 10 primers, primer S147 had the best amplification (Table 3 and Figure 3).

Phylogenetic analysis of *Citrus* varieties

To investigate the phylogenies relationships, statistical analysis was conducted to analyze the RAPD-PCR results. The data show that total 87 locus/alleles were generated by 10 primers listed (Table 3), with an average of 97.7% polymorphic. Cluster analysis was performed by using UPGMA method based on the type 1.0 matrix. Twenty three tested samples were divided to five sub-groups by using genetic similarity index 0.8 as standard (Figure 4), the first sub-group included *C. reticulata* Blanco No.830, *C. reticulata* Blanco xinshengxi No.3 penggan, *C. tangerina* Tanaka, Gongneiyiyuan (Miyauchi Iyokan) and Moketeju (Murcutt tangerine); the second sub-group included *C. sinensis* Osbeck Fulingxiacheng (Valencia orange), *C. sinensis* osbeck tangcheng, *C. sinensis* osbeck hongjiangcheng, *C. sinensis* Osbeck Niuheerqicheng (Newhall Navel orange), *C. sinensis* Osbeck Qingjiaqicheng (Seike Navel orange), Qiuhuijuyou (Fallglo Tangelo) and Nowajuyou (Nova tangelo); the third sub-group included *C. flamea* Hort. ex Tseng shiyueju, *C. flamea* Hort. ex Tseng bayueju and *C. haniana* Hort. ex Tseng Nianju; the fourth sub-group included *C. nobilis* Lour. Xingjinwenzhoumigan (okitsu wase) and *C. nobilis* Lour. gonggan; and the fifth sub-group contain only one accession, C*. junons* Sieb. ex. Tanaka. A genetic similarity matrix was also generated base on the RAPD data (Table 4). The similarity between *C. reticulata* Blanco No.830 and *C. reticulata* Blanco xinshengxi No.3 penggan was 0.955, while the similarity was 0.636, 0.557, 0.557 between Fructus Aurantii and lemon, Fructus Aurantii and Pomelo, Fructus Aurantii and *C. sinensis*, respectively, which were much lower than that described in the foregoing. The similarity among *C. sinensis* breeds was about 0.852 to 0.920. These results

Table 2. RAPD primers selected for PCR analysis.

RAPD primer	Primer sequence (5′-3′)	RAPD primer	Primer sequence (5′-3′)
S8	GTCCACACGG	S227	GAAGCCCAGCC
S10	CTGCTGGGAC	S230	GGACCTGCTG
S28	GTGACGTAGG	S236	ACACCCCACA
S64	CCGCATCTAC	S237	ACCGGCTTGT
S71	AAAGCTGCGG	S238	TGGTGGCGTT
S90	AGGGCCGTCT	S253	GGCTGGTTCC
S92	CAGCTCACGA	S261	CTCAGTGTCC
S99	GTCAGGGCAA	S266	AGGCCCGATG
S147	AGCTGCAGCC	S269	GTGACCGAGT
S154	TGCGGCTGAG	S418	CACCATCCGT
S202	TGAGAGACTC		

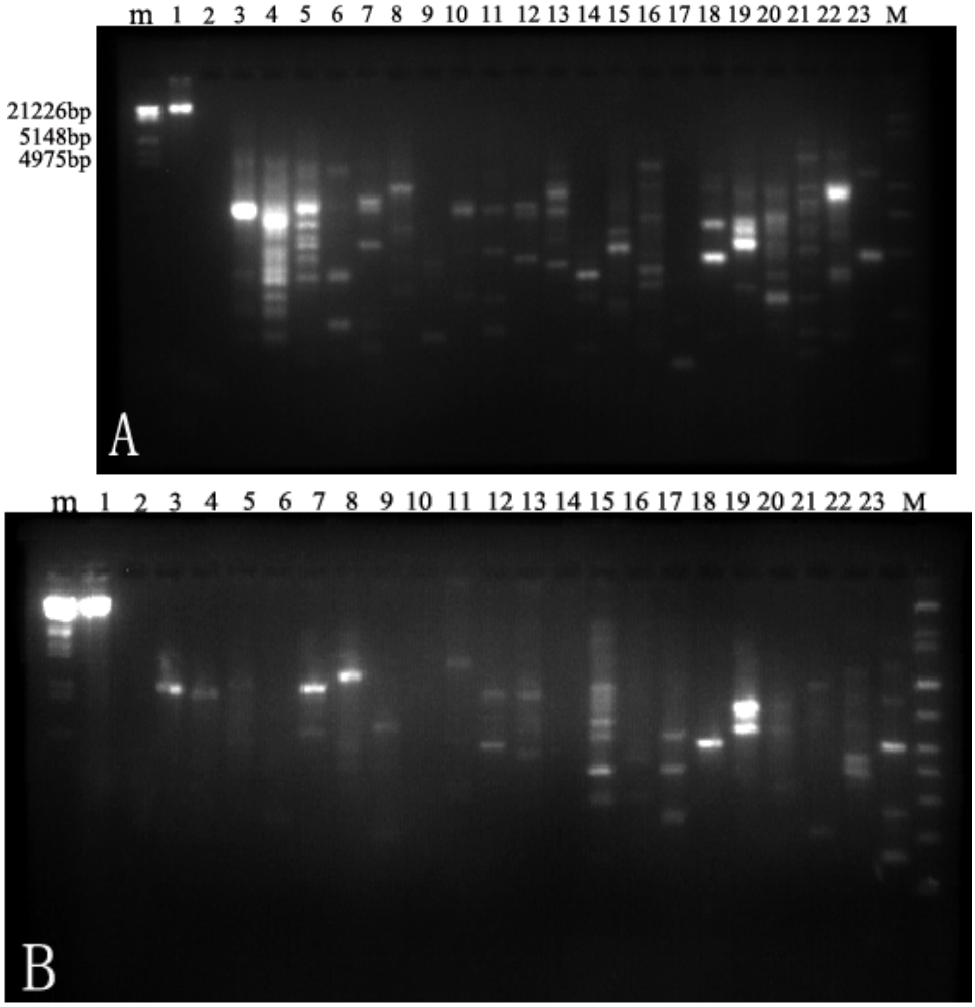

Figure 2. RAPD amplification results in local *Citrus* varieties. m: λDNA/EcoR*I*+Hind*III;* M: marker; A: *C. flamea* Hort. ex Tseng 'shiyueju'; B: *C. flamea* Hort. ex Tseng 'bayueju'; C: *C. flamea* Hort. ex Tseng 'wuyueju'; D: *C. nobilis* Lour. gonggan. Lane 1: Genomic DNA; lane 2: blank control; lane 3: S418; lane 4: S64; lane 5: S237; lane 6: S92; lane 7: S8; lane 8: S238; lane 9:S28; lane 10: S202; lane 11: S10; lane 12: S261; lane 13: S236; lane 14: S269; lane 15: S230; lane 16: S266; lane 17: S253; lane 18: S71; lane 19: S90; 20: lane S147; lane 21: S99; lane 22: S227 and lane 23: S154.

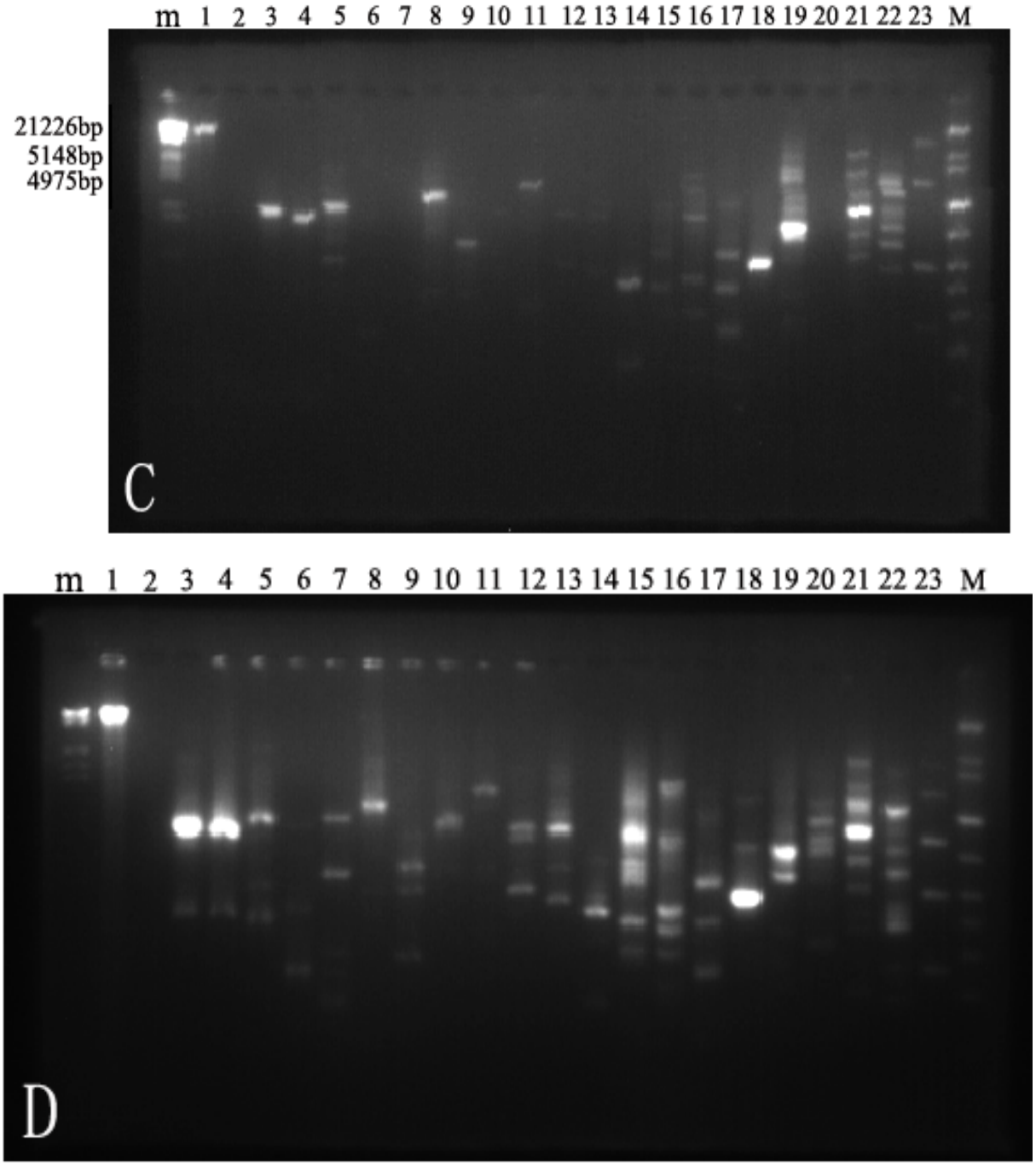

Figure 2. Continue

are consistent with the traditional taxonomy.

C. flamea Hort. ex Tseng shiyueju, *C. flamea* Hort. ex Tseng bayueju and *C. haniana* Hort. ex Tseng Nianju were the special *Citrus* breeds in Guangdong province; in accordance with this, the relationships among them were close according to the dendrogram and genetic similarity. The similarities between *C. nobilis* Lour. gonggan and *C. nobilis* Lour. Xingjinwenzhoumigan (okitsu wase), *C. nobilis* Lour. gonggan and *C. flamea* Hort. ex Tseng shiyueju, *C. nobilis* Lour. Gonggan and *C. haniana* Hort. ex Tseng Nianju were 0.818, 0.841 and 0.784, respectively. *C. nobilis* Lour. gonggan, *C. sinensis* Osbeck Qingjiaqicheng (Seike Navel orange) and *C. sinensis* Osbeck Niuheerqicheng (Newhall Navel orange) had a

Table 3. The primer sequences and PCR amplification results.

Primer	Number of amplified band	Number of polymorphic band	Percentage of polymorphic band
S418	4	4	100
S64	5	5	100
S71	7	7	100
S253	12	12	100
S147	16	16	100
S227	13	12	92
S238	10	9	90
S266	13	12	92
S28	4	4	100
S8	3	3	100

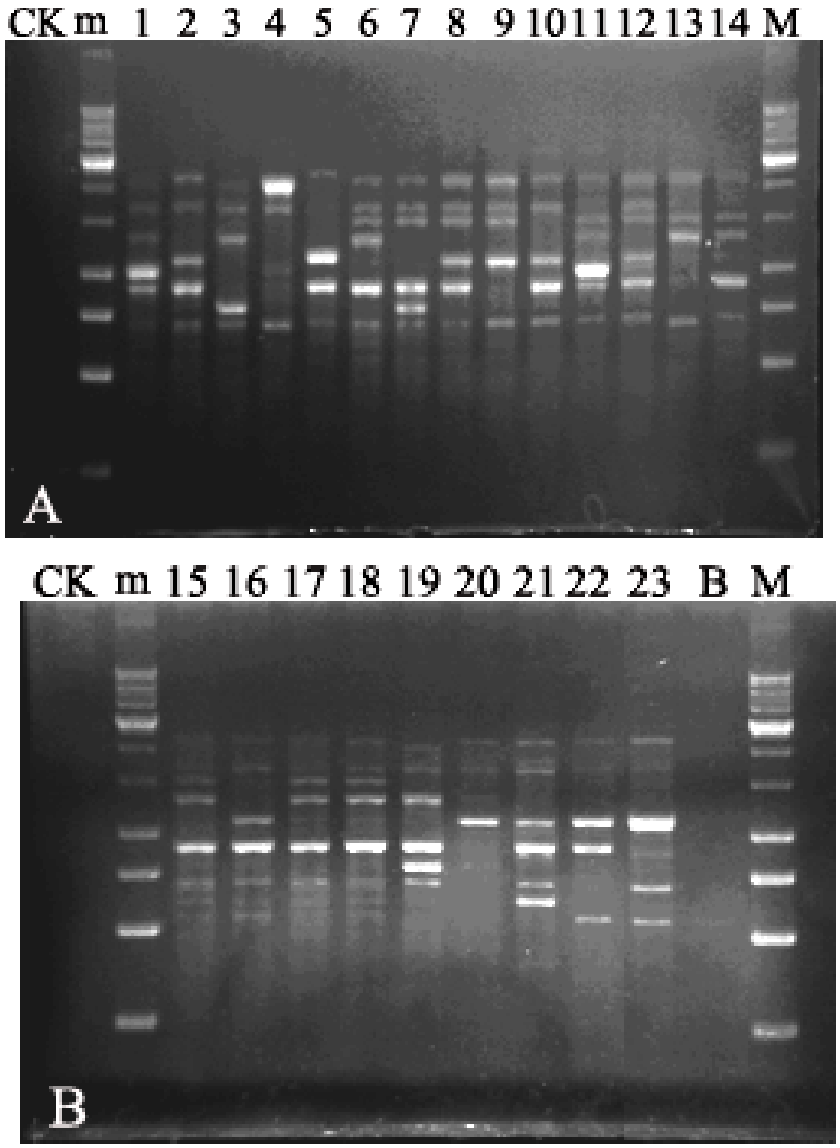

Figure 3. The amplification result of profile of primer S147 on 23 *Citrus* accessions. CK = B: blank control; M: marker; lanes 1 to 23 above the lanes were consistent with the materials numbers listed in Table 1.

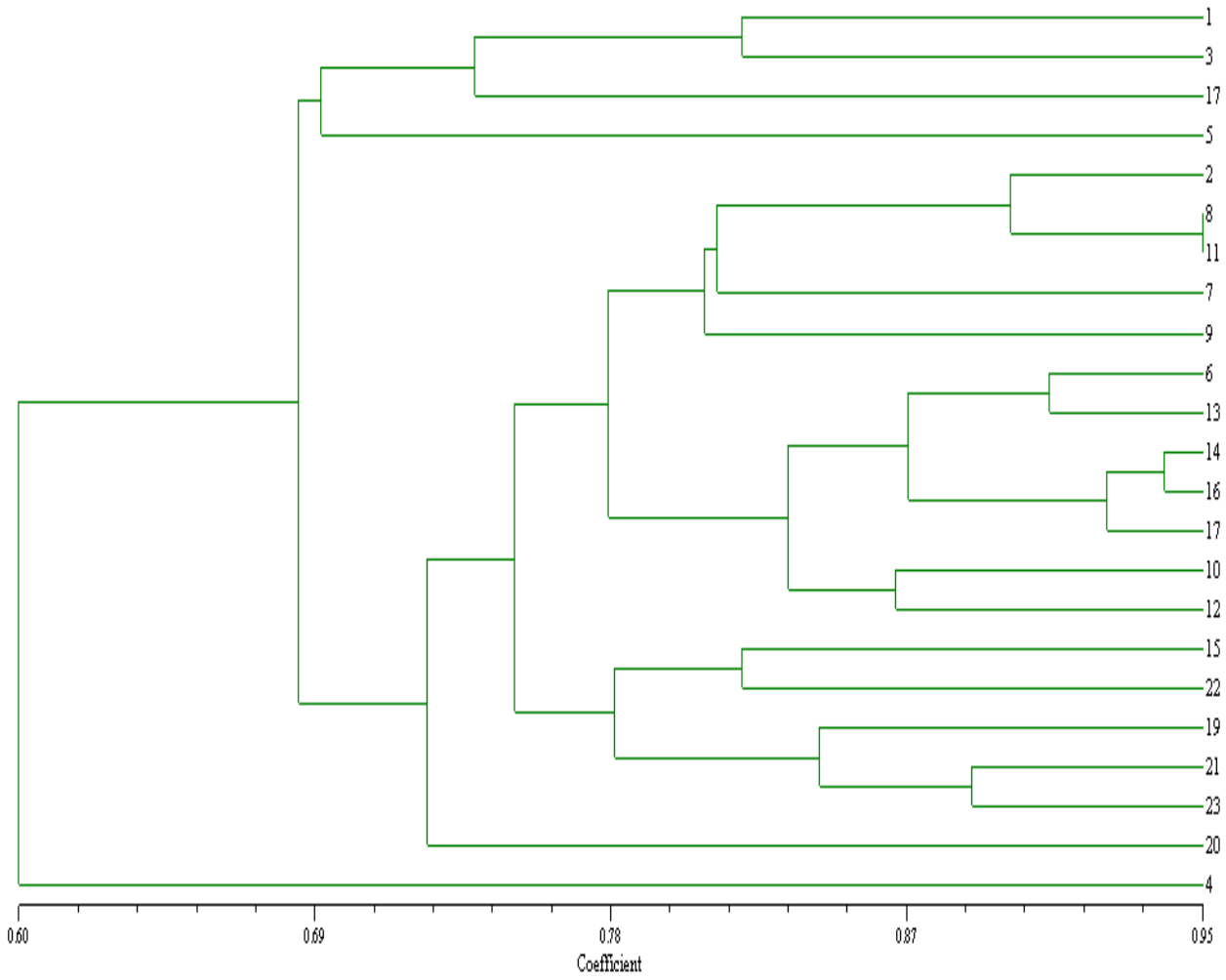

Figure 4. Phylogenetic relationships among 23 *Citrus* accessions based on RAPD data. Numbers 1 to 23 were consistent with the materials numbers listed in Table 1.

close relationship with the genetic similarity of 0.773.

DISCUSSION

In this paper, RAPD technique was efficiently used to identify genetic relationship of Citrus cultivars (Fan et al., 2002; Shi et al., 1998; Chen et al., 2006), however, the PCR reaction and conditions were not unanimous (Chen et al., 2006; Xiao et al.; Clark, 1998; Dieffenbach, 1998; Liu et al., 2006). In order to eliminate these controversies, we optimized each of the RAPD components' concentration and conducted the gradient PCR. We created the optimized *Citrus* RAPD-PCR reaction: 10 ng DNA as template, 0.2 μmol·L^{-1} primer, 0.2 mmol·L^{-1} dNTPs, 0.1 U·μl^{-1}Taq, 2. 75 mmol·L^{-1} Mg^{2+} and the PCR program was as follows: 94℃ for 3 min; 94℃ for 1 min, 44℃ for 90 s, 73℃ for 2 min and was repeated in 36 cycles; then 72℃ for 10 min. The genetic similarity among all the 23 citrus accessions tested ranges from 0.534 to 0.955, indicating a wide genetic background. A total of 87 locus/alleles were generated by 10 primers; each primer generated 8.4 bands with an average of 97.7% polymorphic. According to the cluster analysis, Fructus Aurantii, lemon, Pomelo and *Citrus* (mandarin, tangerines, orange) were placed in different groups which was consistent with the traditional taxonomy. Meanwhile, lines of *C. reticulata* Blanco (*C. reticulata* Blanco No.830, *C. reticulata* Blanco xinshengxi No.3 penggan, *C. tangerina* Tanaka, Gongneiyiyuan (Miyauchi Iyokan) could be distinguished by RAPD and also clustered in the same group indicating that RAPD was suitable for the identification of *Citrus* species, accessions and lines.

The genetic similarity between *C. nobilis* Lour. Gonggan and *C. flamea* Hort . ex Tseng shiyueju was 0.818 respectively which was compared with other accessions tested and *C. flamea* Hort . ex Tseng shiyueju was a famous local cultivated breed in domestic orange planting with a long cultivated history in sihui orgion. There may be some phylogenetic relationship for both of them. Gan et

Table 4. Genetic similarity of 23 *Citrus* accessions.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1.000																						
2	0.773	1.000																					
3	0.818	0.659	1.000																				
4	0.716	0.625	0.648	1.000																			
5	0.716	0.693	0.716	0.636	1.000																		
6	0.807	0.761	0.693	0.636	0.704	1.000																	
7	0.727	0.818	0.659	0.580	0.670	0.807	1.000																
8	0.750	0.909	0.636	0.602	0.670	0.784	0.818	1.000															
9	0.716	0.784	0.648	0.614	0.682	0.841	0.807	0.830	1.000														
10	0.761	0.761	0.625	0.681	0.636	0.864	0.784	0.784	0.818	1.000													
11	0.773	0.886	0.636	0.625	0.670	0.807	0.795	0.955	0.807	0.807	1.000												
12	0.761	0.830	0.716	0.636	0.705	0.864	0.830	0.852	0.818	0.864	0.830	1.000											
13	0.807	0.784	0.693	0.614	0.659	0.909	0.761	0.807	0.795	0.818	0.807	0.864	1.000										
14	0.784	0.739	0.739	0.591	0.636	0.864	0.716	0.761	0.750	0.818	0.761	0.864	0.886	1.000									
15	0.716	0.761	0.670	0.568	0.659	0.773	0.693	0.761	0.727	0.705	0.739	0.773	0.750	0.818	1.000								
16	0.795	0.727	0.727	0.557	0.670	0.875	0.727	0.750	0.739	0.784	0.750	0.830	0.852	0.943	0.807	1.000							
17	0.750	0.727	0.705	0.580	0.625	0.875	0.727	0.773	0.761	0.784	0.773	0.830	0.852	0.920	0.852	0.931	1.000						
18	0.704	0.591	0.773	0.557	0.648	0.716	0.636	0.614	0.602	0.625	0.614	0.670	0.693	0.739	0.693	0.773	0.750	1.000					
19	0.772	0.841	0.750	0.580	0.670	0.761	0.727	0.795	0.739	0.739	0.773	0.807	0.739	0.761	0.784	0.773	0.750	0.659	1.000				
20	0.659	0.773	0.545	0.534	0.648	0.739	0.727	0.750	0.7389	0.739	0.750	0.716	0.693	0.693	0.739	0.682	0.705	0.591	0.682	1.000			
21	0.739	0.830	0.693	0.568	0.705	0.773	0.693	0.784	0.795	0.727	0.761	0.773	0.773	0.773	0.841	0.784	0.784	0.625	0.875	0.761	1.000		
22	0.739	0.761	0.675	0.568	0.705	0.773	0.670	0.716	0.705	0.727	0.716	0.773	0.773	0.773	0.818	0.761	0.761	0.648	0.807	0.670	0.818	1.000	
23	0.693	0.784	0.625	0.568	0.636	0.727	0.648	0.716	0.750	0.705	0.739	0.727	0.705	0.682	0.727	0.670	0.670	0.534	0.807	0.761	0.886	0.705	1.000

al. (2008), Zeng (1960) and Tanaka (1996, 1997) suggested that *C. nobilis* Lour. Gonggan is one of *C. reticulate* Blanco basing on morphology; while Zhou and Ye (2009) proposed that *C. nobilis* Lour. Gonggan belong to *C. nobilis* based on its cultivated characteristics. In this paper, we applied RAPD technique to analyze *Citrus* species. Based on RAPD results, we generated phylogenic tree of citrus species and genetic relationship between *C. nobilis* Lour. Gonggan and other *Citrus* species. *C. nobilis* Lour. Gonggan and *C. nobilis* Lour. Xingjinwenzhoumiganin clustered in the same group with the similarity of 0.818, supporting the view that *C. nobilis* Lour. Gonggan was one of the subspecies in *C. nobilis*. It was recorded that *C. nobilis* Lour. Gonggan is a cross species between tangerine and orange, however, its cross parents remains unknown. In the present research, the dendrogram analysis indicated that *C. nobilis* Lour. Gonggan, almost all the tangerine and orange clustered in one big group which support the view that *C. nobilis* Lour. Gonggan is a tangerine × orange cross species, moreover, *C. nobilis* Lour. Gonggan, *C. haniana* Hort. ex Tseng Nianju and *C. flamea* Hort. ex Tseng shiyueju clustered in one sub-group, implying that *C. haniana* or *C. flamea* was one of the most probable cross parents of *C.*

nobilis Lour. Gonggan, however, the other cross parent remains unknown. In future, we will analyze the other parent of Gonggan based on mitochondrial and chloroplast gene sequences.

Conclusions

Based on RAPD data, three conclusions were drawn: 1) An optimized RAPD-PCR reaction system for *Citrus* species was developed. The PCR program was as follows: 94°C for 3 min; 94°C for 1 min, 44°C for 90 s, 73°C for 2 min and were repeated in 36 cycles; then, 72°C for 10 min. 2) RAPD is a fast and efficient method to study *Citrus* species phylogenetic relationship. 3) *C. haniana and C. flamea* were the most probable cross parents of *C. nobilis* Lour. Gonggan.

ACKNOWLEDGEMENTS

We are thankful to the Science and Technology Planning Project of Guangdong Province (2010B080201006) and Science and Technology Planning Project of Zhaoqing City (2009N029) for their financial support.

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