Genetic background of *Citrus nobilis* Lour. 'Gonggan' based on the chloroplast trnL gene

Y.-J. Guo[1], Q.-H. Ji[1], X.-Q. Zhou[1], Y.-P. Hu[1], L.-Y. Guo[1], H. Jiang[1] and J.-W. Zeng[2]

[1]Fruit Tree Research Institute, Zhaoqing University, Guangdong, China
[2]Fruit Tree Research Institute,
Guangdong Province Academy of Agricultural Sciences, Guangdong, China

Corresponding author: Q.-H. Ji
E-mail: qhgee@hotmail.com

ABSTRACT. The genetic backgrounds of many *Citrus* varieties are quite complex. Classifications and phylogenetic relationships of *Citrus* species have become the focus of researchs. Some conserved genes of chloroplast genome's research have been proven effective in determining the biosources of hybrids and phylogenetic analysis. Thus, we studied variations among the chloroplast trnL gene sequences of 10 *Citrus* species, including *C. nobilis* Lour. 'Gonggan'. The amplification results of different trnL target genes and identification of the double-enzyme cut after cloning show that lengths of all trnL sequences were within 895 to 935 bp and a total of 24 variation sites were detected among the 10 material samples. Clustering analysis revealed differences in trnL genes caused by systematic evolution and allowed the determination of variations among *Citrus* varieties. Variation sites of trnL sequences can be used in the phylogenetic classification and species identification of *Citrus*, and the results agreed with

random amplified polymorphic DNA marker results. *C. nobilis* Lour. 'Gonggan' is closely associated with the other two varieties in Zhaoqing area, and *C. nobilis* Lour. 'Gonggan' and *C. haniana* Hort. ex Tseng 'Sihuihanggan' can be classified into the same category. *C. nobilis* Lour. 'Gonggan' as a natural hybrid is probably a hybrid with *C. haniana* Hort. ex Tseng 'Sihuihanggan' as its female parent.

Key words: *Citrus nobilis* Lour. 'Gonggan'; trnL; *Citrus*; Genetic background; Genetic relationship

INTRODUCTION

The classifications and phylogenetic relationships of *Citrus* species have been debated within academic circles ever since *Citrus* was defined in 1753 by the Swedish scientist Carolus Linnaeus. Thus far, systematic research on the classifications and genetic origins of *Citrus* plants has consisted of morphological and cytological studies, numerical classification, molecular markers, and so on, with many foreign and domestic scholars achieving a certain extent of progress (Xie et al., 2008; Ji and Guo, 2011). Several analytical methods for studying the systematic evolutions of conserved genes (e.g., rbcL) have been proven effective in determining the biosources of hybrids because chloroplast genomes are inherited from the female parent (Green et al., 1986; Wang and Jiang, 2010).

Citrus can easily undergo cross-breeding and open pollination, so natural hybrid varieties such as *C. nobilis* Lour. 'Gonggan' exist everywhere. Thus, the genetic backgrounds of many *Citrus* varieties are quite complex. While the morphological characteristics of some *Citrus* varieties are distinctly different, their genetic relationships may be very closely related. Studies on the chloroplast genomes of *Citrus* varieties can provide solid evidence regarding their genetic structures and classification statuses. In addition, they can also generate meaningful guidance for further cultivation of improved varieties.

C. nobilis Lour. 'Gonggan' has an orange-like shape and a thin golden peel. It is fragrant, tastes like honey, has an exterior similar to oranges, and is easy to peel like tangerines. *C. nobilis* Lour. 'Gonggan' is a highly productive and local characteristic species in farm lands of the ZhaoQing region. It was chosen as a tribute fruit for the imperial family during the Ming and Qing Dynasties because of its excellent quality. It is currently the second most-cultivated fruit in the ZhaoQing region, with sugar tangerines being the first. Deqing County in the ZhaoQing region is one of the main cultivation sites of *C. nobilis* Lour. 'Gonggan'. According to the Sihui County Record from the Qing Dynasty, the fruit originated from the Sihui region of ZhaoQing and is a natural hybrid of orange and tangerine. However, the species of its parents can no longer be determined. The Sihui region historically possesses rich *Citrus* resources; over 20 cultivars or more than 30 species of *Citrus* have been planted in the Sihui region. *C. nobilis* Lour. 'Gonggan' is believed to have relationships with several historical plant species (Ji et al., 2010).

The present study determines variations among the trnL gene sequences of 10 *Citrus* species, including *C. nobilis* Lour. 'Gonggan', to provide molecular evidence of the genetic background of *Citrus* and the origin of *C. nobilis* Lour. 'Gonggan'.

MATERIAL AND METHODS

Ten test material samples were prepared. Samples included *C. nobilis* Lour. 'Gonggan', *C. haniana* Hort. ex Tseng 'Sihuihanggan', *Citrus flamea* Hort. ex Tseng 'Shiyueju', *Citrus sinensis* Osbeck 'Lanhuacheng', *Atalantia buxifolia* (Poir.) Oliv., *Citrus grandis* (L.) Osbeck 'Guanximiyou', *Poncirus trifoliata* (L.) Raf, *Citrus limon* (L.) Burm. f. 'Youlikeningmeng' (Eureka lemon), *Citrus sinensis* Osbeck 'Niuheerqicheng' (Newhall navel orange), and *Citrus nobilis* Lour. 'Xingjinwenzhoumigan' (Okitsu wase). The suspected parent of *C. nobilis* Lour. 'Gonggan' (*C. haniana* Hort. ex Tseng 'Sihuihanggan') is the dominant species currently cultivated in the Zhaoqing region (*C. nobilis* Lour. 'Gonggan' and *C. flamea* Hort. ex Tseng 'Shiyueju').

DNA extraction was performed as follows: 1) young and healthy *Citrus* lamina were selected; 2) genomes were extracted using the modified hexadecyltrimethylammonium bromide (CTAB) method (Ji and Guo, 2011); 3) DNA purity and concentration were measured by 1% agarose gel electrophoresis and a micro-spectrophotometer; 4) 100 ng DNA was obtained according to the DNA concentration to be used as polymerase chain reaction (PCR) template.

Amplification and clone and sequence analyses of trnL target bands utilized the following trnL amplification primers: TrnlP5: 5'-TAAAAATGGGTAATCCTGAGCCAA-3' and TrnlP3: 5'-CCAGCTGAGCTACCCGAC-3'. The amplification protocol was 94°C for 3 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

The PCR outcome was tested by 1.5% agarose gel electrophoresis. Some of the gel slices with special target bands displayed in the electrophoretogram were recovered and submitted to T-A cloning. The PCR outcome was connected to the T Carrier (pMD19T, Takara). Recombinants were continuously transformed into the Top 10 competent cells. Cells were revived at 37°C for 40 min and spread on a plate (Luria-Bertani medium with ampicillin). The plate was placed upside down overnight at a constant temperature for cultivation. PCR amplification was performed using the DNA of single-colony plasmids as template. The amplification protocol was as follows: 94°C for 3 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

The bacterial colony with the highest positive value was plated and cultivated overnight. At least 10 positive clones in each test material sample were selected and tested with the Takara incision enzyme by identification of the double-enzyme cut. Positive clones were screened and sent to Shanghai Bioengineering Co., Ltd. for bi-directional sequencing analysis.

RESULTS

Genome electrophoresis results of the 10 material samples are shown in Figure 1. Amplification results of different trnL target genes and identification of the double-enzyme cut after cloning are presented in Figure 2. Sequencing and positioning/ranking analyses are shown in Table 1.

Lengths of all trnL sequences were within 895 to 935 bp (Table 2). A total of 24 variation sites were detected among the 10 material samples. The basic group variation types

included transformations of A-G, T-C, and C-T, and transversion of C-A, T-G, G-T, C-G, A-T, and A-C. Deficiency variations were observed in three regions. Insertion variations with simple reduplications of GCAATA and TTTTTC were also observed in different varieties.
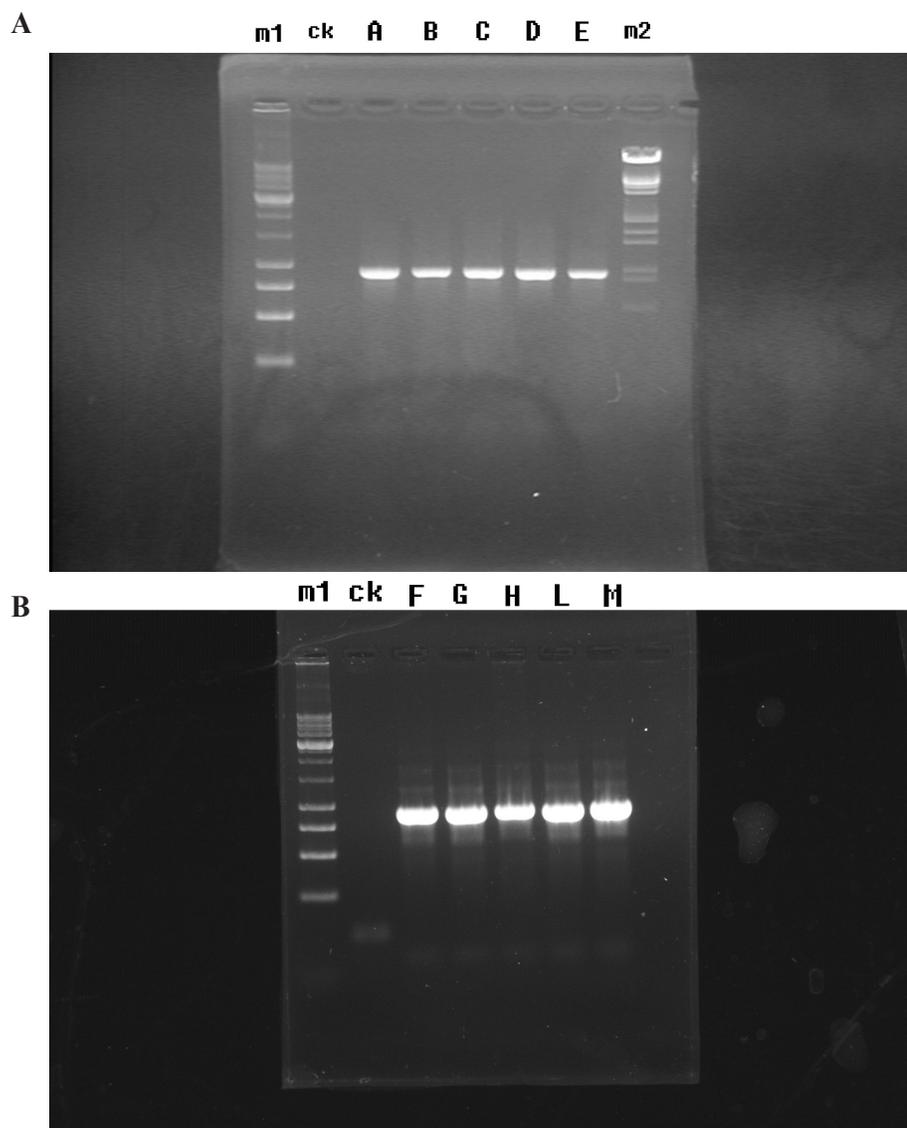
Figure 1. trnL gene amplification of the 10 *Citrus* species. **A.** *Lane m1* = 1-kb marker; *lane ck* = PCR-negative control; *lanes A, B, C, D, E*, and *m2* = λDNA/*Eco*RI+*Hin*dIII. **B.** *Lane m1* = 1-kb marker; *lane ck* = PCR-negative control; *lanes F, G, H, L*, and *M*. Lane A = *Citrus nobilis* Lour. 'Gonggan'; *lane B = C. haniana* Hort. ex Tseng 'Sihuihanggan'; *lane C = C. flamea* Hort. ex Tseng 'Shiyueju'; *lane D = C. sinensis* Osbeck 'Lanhuacheng'; *lane E = Atalantia buxifolia* (Poir.) Oliv.; *lane F = C. grandis* (L.) Osbeck 'Guanximiyou'; *lane G = Poncirus trifoliata* (L.) Raf; *lane H = C. limon* (L.) Burm. f. 'Youlikeningmeng'; *lane L = C. sinensis* Osbeck 'Niuheerqicheng'; *lane M = C. nobilis* Lour. 'Xingjinwenzhoumigan'.

A

B

C

D

E
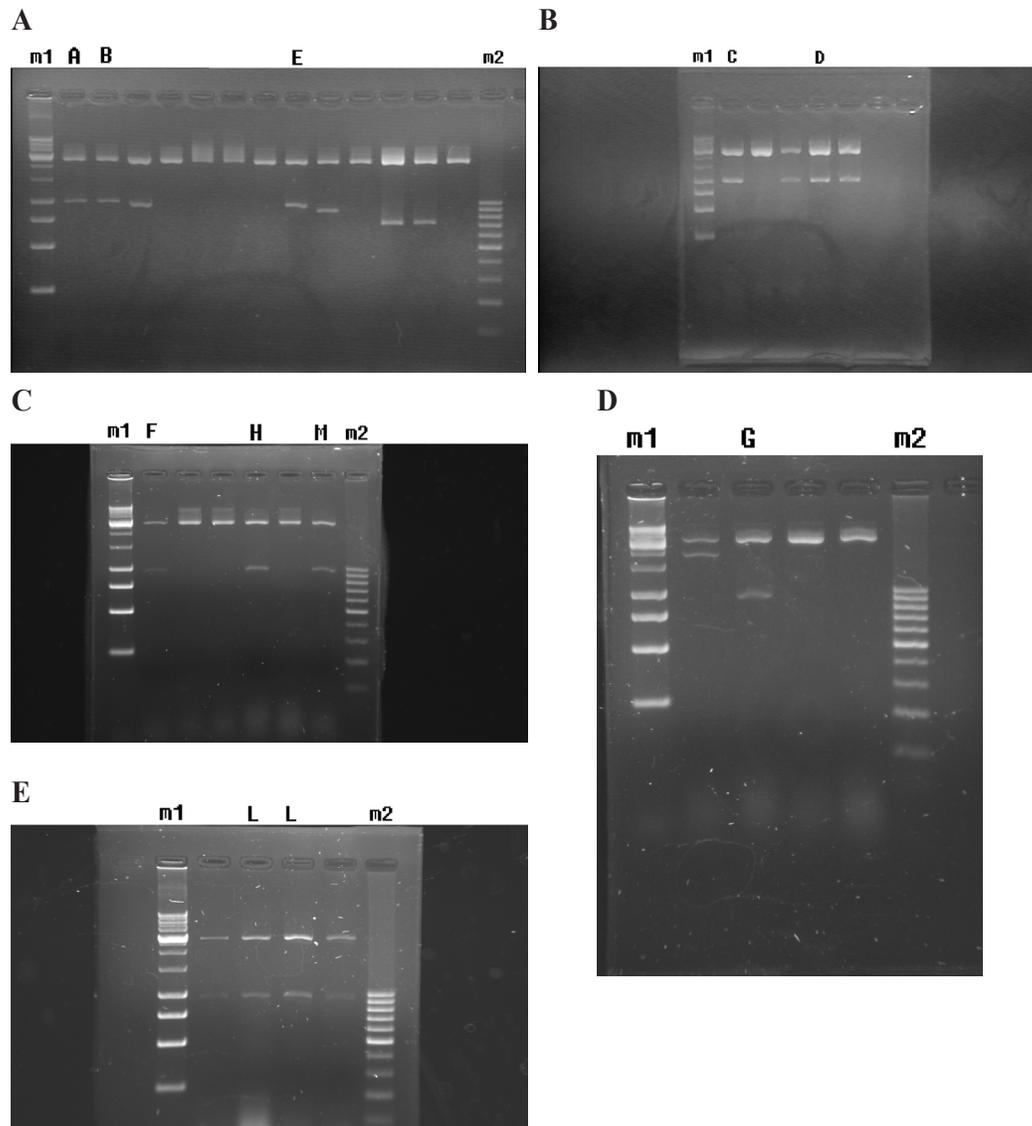
Figure 2. Double-enzyme cut electrophoresis after cloning trnL sequences of the 10 *Citrus* species. *Lane m1* = 1-kb marker; *lane m2* = 100-bp marker; *lanes A* to *M* = successful sample about construction of cloning vector and sequencing

The dendrogram (Figure 3) reveals that *C. nobilis* Lour. 'Gonggan' and *C. haniana* Hort. ex Tseng 'Sihuihanggan' can be classified in the same category. This category also includes *C. flamea* Hort. ex Tseng 'Shiyueju' and *C. nobilis* Lour. 'Xingjinwenzhoumigan'. The genetic relationships between other species and the results of their typical morphological classifications showed some similarities. Results of this study are similar to the random amplified polymorphic DNA (RAPD) results previously reported.

**Table 1.** Variance analysis of trnL sequences of the 10 *Citrus* species.

	1-28 (5'-3')	79	83	165-170
Standard sequence	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-F	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-G	-	T	T	-
trnL-H	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-L	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-M	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-A	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-B	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-C	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-D	CCAGCTGAGCTATCCCGACCATTTCCGA	T	C	-
trnL-E	CCAGCTGAGCTATCCCGACCATTTCCGA	G	A	GCAATA

	207	213-221	296	358	369	584
Standard sequence	C	GGTGTTCAT	A	T	G	T
trnL-F	C	GGTGTTCAT	A	T	G	T
trnL-G	C	GGTGTTCAT	G	T	G	T
trnL-H	C	GGTGTTCAT	A	T	G	T
trnL-L	C	GGTGTTCAT	A	C	G	T
trnL-M	C	GGTGTTCAT	A	T	G	T
trnL-A	A	GGTGTTCAT	A	T	G	T
trnL-B	A	GGTGTTCAT	A	T	G	T
trnL-C	A	GGTGTTCAT	A	T	G	T
trnL-D	C	GGTGTTCAT	A	T	G	T
trnL-E	C	-	A	T	T	C

	586	614	622	639-644	651	681
Standard sequence	A	T	A	ATAACA	-	T
trnL-F	A	T	A	ATAACA	-	T
trnL-G	T	T	A	-	-	T
trnL-H	A	T	A	ATAACA	-	T
trnL-L	A	T	A	ATAACA	-	T
trnL-M	A	T	A	ATAACA	TTTTTC	T
trnL-A	A	T	A	ATAACA	TTTTTC	T
trnL-B	A	T	A	ATAACA	TTTTTC	T
trnL-C	A	T	A	ATAACA	TTTTTC	T
trnL-D	A	T	A	ATAACA	-	T
trnL-E	A	G	C	-	-	G

	689	721	745	787-788	790	821	829
Standard sequence	T	A	T	TT	C	A	T
trnL-F	C	A	T	TG	C	A	T
trnL-G	T	A	T	TT	C	A	T
trnL-H	T	A	T	TG	C	A	T
trnL-L	T	A	T	CG	C	G	T
trnL-M	T	C	T	TT	C	A	T
trnL-A	T	C	T	TT	C	A	T
trnL-B	T	C	T	TT	C	A	C
trnL-C	T	C	T	TT	C	A	T
trnL-D	T	A	T	TG	C	A	T
trnL-E	T	A	G	TT	G	A	T

A = *Citrus nobilis* Lour. 'Gonggan'; B = *C. haniana* Hort. ex Tseng 'Sihuihanggan'; C = *C. flamea* Hort. ex Tseng 'Shiyueju'; D = *C. sinensis* Osbeck 'Lanhuacheng'; E = *Atalantia buxifolia* (Poir.) Oliv.; F = *C. grandis* (L.) Osbeck 'Guanximiyou'; G = *Poncirus trifoliata* (L.) Raf; H = *C. limon* (L.) Burm. f. 'Youlikeningmeng'; L = *C. sinensis* Osbeck 'Niuheerqicheng'; M = *C. nobilis* Lour. 'Xingjinwenzhoumigan'.

Table 2. trnL basic group contents of the 10 *Citrus* species.

	trnL-F	trnL-G	trnL-H	trnL-L	trnL-M	trnL-A	trnL-B	trnL-C	trnL-D	trnL-E
Base number	929	895	929	929	935	935	935	935	929	920

A-M = varieties as defined in legend to Table 1.
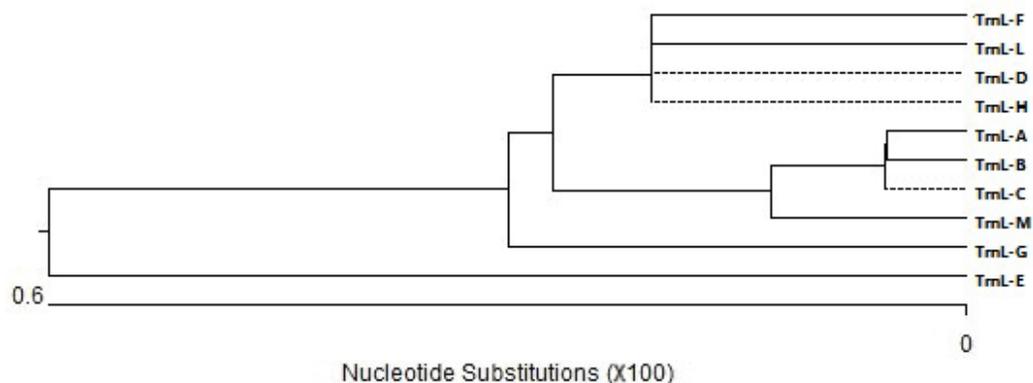
Figure 3. Dendrogram for the 10 *Citrus* species derived from cluster analysis of trnL sequences. A-M = varieties as defined in legend to Figure 1.

DISCUSSION

In recent years, *Citrus* species have been commonly identified using RAPD, simple sequence repeats, amplified fragment length polymorphism, and similar molecular marker methods. Many studies on the genetic relationships and hybrid origins of *Citrus* species have also been carried out. These studies have provided useful information for the classification and identification of *Citrus*. However, information about the chloroplast genomes of *Citrus* remains limited.

The trnL gene is a gene for encoding leucine tRNA in the chloroplast genomes. Its application in studies of phylogeny and inter-species/species identification has been proven to be effective in short-tube lycoris (Taberlet et al., 1991; Li and Lu, 2005; Yuan et al., 2008). In the present study, we selected lemon, pomelo, Mandarin orange, tangerine, orange, Poncirus, and Atalantia as materials for comparison to construct a phylogeny tree. Clustering results showed that *C. nobilis* Lour. 'Gonggan', *C haniana* Hort. ex Tseng 'Sihuihanggan', and *C. flamea* Hort. ex Tseng 'Shiyueju' belong to the same category. This category also included *C. nobilis* Lour. 'Xingjinwenzhoumigan'. *A. buxifolia* (Poir.) Oliv. and *P. trifoliata* (L.) Raf belonged to different categories.

The classifications of most species showed that the trnL molecule systematically possesses similarities with typical morphological classification methods. Results are also similar to the dendrogram of *Citrus* classification acquired by the current authors using RAPD molecular marker methods (Ji et al., 2011). Differences in trnLs generated through systematic evolution can be identified, and these differences represent variations between *Citrus* species. Thus, the variation sites of trnL sequences can be used in the genealogical classification and species identification of *Citrus*.

According to farmers from the Sihui County, *C. haniana* Hort. ex Tseng 'Sihuihanggan' is the biological parent of *C. nobilis* Lour. 'Gonggan', which has a wide circulation. *C. haniana* Hort. ex Tseng 'Sihuihanggan' was already a cultivar before *C. nobilis* Lour. 'Gonggan' and *C flamea* Hort. ex Tseng 'Shiyueju' became major varieties in the ZhaoQing area. In addition, *C. haniana* Hort. ex Tseng 'Sihuihanggan' is nearly extinct (Ji et al., 2010). Signif-

icant efforts have been made to collect and preserve *C. haniana* Hort. ex Tseng 'Sihuihanggan'. Different citrus varieties were considered the main cultivar during different historical periods in the ZhaoQing area. Today, *Citrus* cultivars in the ZhaoQing area are fresh cultivars, and are rarely stored and processed. *C. haniana* Hort. ex Tseng 'Sihuihanggan' ripens every December, requiring time to sweeten after the Spring Festival. Therefore, it is not suitable for the current *Citrus* industry in the local area and is gradually being eliminated from the market.

The trnL sequence comparison between *C. nobilis* Lour. 'Gonggan' and *C. haniana* Hort. ex Tseng 'Sihuihanggan' reveals that their sequences are mostly identical except for a variation at 829 bp, in which a T/C base transition occurs. This site can be used to differentiate *C. nobilis* Lour. 'Gonggan' from *C. haniana* Hort. ex Tseng 'Sihuihanggan'. The chloroplast genome follows maternal inheritance. Therefore, *C. nobilis* Lour. 'Gonggan' could be a hybrid with *C. haniana* Hort. ex Tseng 'Sihuihanggan' as its female parent. *C. haniana* Hort. ex Tseng 'Sihuihanggan' is of greater importance compared to *C. nobilis* Lour. 'Gonggan' in terms of theoretical and practical significance in breeding based on storage traits, flavor, and quality changes after storage. The species can serve as a storable variety for breeding and postharvest physiological studies. *C. nobilis* Lour. 'Gonggan' may also be an appropriate test material for pathological studies on *Citrus* anthracnose as a sensitive anthracnose variety.

The standard trnL sequences of *C. nobilis* Lour. 'Gonggan', *C. haniana* Hort. ex Tseng 'Sihuihanggan', *C. flamea* Hort. ex Tseng 'Shiyueju', and *C. nobilis* Lour. 'Xingjinwenzhoumigan' as Sinocitrus tseng are interposed by a short TTTTTC repeated sequence at the 651-bp site. This repeated sequence is not found in the standard trnL sequences of other tested varieties, indicating that this site can be considered a key site in distinguishing Sinocitrus tseng from other species. *C. nobilis* Lour. 'Gonggan' and *C. flamea* Hort. ex Tseng 'Shiyueju' have different morphologies, but their trnL sequences reveal that they have a close genetic relationship. *C. nobilis* Lour. 'Gonggan', *C. haniana* Hort. ex Tseng 'Sihuihanggan', and *C. flamea* Hort. ex Tseng 'Shiyueju' are the local cultivars of the ZhaoQing area during different historical stages. They all have the same trnL sequences of 207 bp, a feature that other varieties do not possess. C-A transversion occurs in these three varieties compared to other *Citrus* varieties.

CONCLUSIONS

C. nobilis Lour. 'Gonggan', *C. haniana* Hort. ex Tseng 'Sihuihanggan', and *C. flamea* Hort. ex Tseng 'Shiyueju' are the local cultivars of the ZhaoQing area during different historical stages and have close genetic relationships. *C. nobilis* Lour. 'Gonggan' is probably a hybrid with *C. haniana* Hort. ex Tseng 'Sihuihanggan' as its female parent.

Differences in trnL genes caused by systematic evolution allow the determination of variations between *Citrus* varieties. Variation sites of trnL sequences can be used in the phylogenetic classification and species identification of *Citrus*.

ACKNOWLEDGMENTS

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