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Molecular Authentication of the Chinese Herb Huajuhong and Related Medicinal Material by DNA Sequencing and ISSR Markers

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ABSTRACT

DNA sequences in the chloroplast (*trn*H-*psb*A intergenic spacer) and nuclear (ITS) regions were amplified and determined for Huajuhong derived from the peels of the immature fruits of *Citrus grandis* 'Tomentosa' and related medicinal material. These sequences together with the inter-simple sequence repeats (ISSR) markers may be used to differentiate *C. grandis* 'Tomentosa' from other *Citrus* variants for the prevention of misuse.

Key words**:** pomelo peel, *Citrus*, Rutaceae, authentication, *trn*H-*psb*A, intergenic spacer (ITS), inter-simple sequence repeats (ISSR)

INTRODUCTION

The Chinese herb Huajuhong (*Exocarpium Citri grandis*), according to the 2005 edition of the Pharmacopoeia of the People's Republic of China, is the peel from the immature fruits of both a hairy cultivar and the common form of pomelo. The source plants, described in that Pharmacopoeia, are, respectively, *Citrus grandis* Osbeck 'Tomentosa' and *Citrus grandis* Osbeck. The correct scientific names, however, should be *Citrus maxima* (Burm.) Merr. 'Tomentosa' and *Citrus maxima* (Burm.) Merr., respectively[1-4].

Huajuhong is a popular Chinese medicinal material for the relief of tussis and phlegm symptoms. Peel of the hairy cultivar is traditionally regarded as superior with stronger antitussive function[5,6] and thus commands a much higher market value. It differs from the common form in having densely tomentose fruit wall. The best production area of the cultivar is Huazhou city of Guangdong Province of China, so the fruit is called Huazhouyou in Chinese, while the common form is called You (HZY and YO, respectively, hereafter).

In many herbal markets, YO which constitutes of different *Citrus* species, is often used as substitute or adulterant of HZY. The commercial pomelo peels are always in shredded slices. The dense hairy fruit wall of HZY is not a reliable character to differentiate these two commodities. On the other hand, the usefulness of chemical methods is limited since the different growth condition, storage condition, age of the sample, and processing and extracting method of the Exocarpium Citri grandis all affect the result[7,8]. Therefore, a reliable authentication method for *Citrus* species is essential for the prevention of misuse. Recently, DNA techniques have been developed in the area of phylogeny and authentication studies between closely related species. For examining the relationship among the *Citrus* genera, RAPD [9], SSR[10,11], ISSR[9,12], SRAPs [13], nuclear DNA[14,15] and chloroplast DNA[4,16,17] have been used. By Random Amplified Polymorphic DNA (RAPD), it has been shown that sexual reproduction and the changes of production areas can result in the genetic diversity of *Citrus grandis* "Tomentosa"[18]. It has also been reported that there are some minor differences in the ITS sequences between

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Table 1. Samples used in this study and GenBank accession number of the DNA sequences.

Number	Species	Sample Code	Place for collection	GenBank accession no.
1-5	*Citrus grandis* 'Tomentosa'	HZY-01 ~ HZY-05	Qinghu, Luchuan, Guangxi, China	
6-11	*C. grandis* 'Tomentosa'	HZY-06 ~ HZY-11	Daling, Pingding, Huazhou, Guangdong, China	
12	*C. grandis* 'Tomentosa'	HZY-12	Guangzhou University of Chinese Medicine, Guangdong, China	ITS: GQ231956- GQ231962 *trn*H-*psb*A: GQ267053-GQ267058, GQ267065 (Note: These no. cover 1-15)
13	*C. grandis* 'Tomentosa'	HZY-13	Southern Medical University, Guangdong, China	
14	*C. grandis* 'Tomentosa'	HZY-14	Qinghu, Luchuan, Guangxi, China	
15	*C. grandis* 'Tomentosa'	HZY-15	Qinghu, Luchuan, Guangxi, China	
16	*Citrus grandis* Osbeck	YO-01	Mei country, Guangdong, China	
17	*C. grandis* Osbeck	YO-02	Yulin, GuangXi, China	ITS: GQ231963- GQ231965 *trn*H-*psb*A: GQ267059-GQ267061 (Note: These no. cover 16-19)
18	*C. grandis* Osbeck	YO-03	Guangzhou, Guangxi, China	
19	*C. grandis* Osbeck	YO-04	Jiangkou, Guiping, Guangxi, China	
20	*Citrus chachiensis*	GAN	Guangzhou University of Chinese Medicine, Guangdong, China	ITS: GQ231966 *trn*H-*psb*A: GQ267062
21-22	*Citrus reticulata*	JU-01-JU-02	Guangzhou University of Chinese Medicine, Guangdong, China	ITS: GQ231967 *trn*H-*psb*A: GQ267063
23	*Citrus medica* var. *sarcodactylis*	FO	Guangzhou University of Chinese Medicine, Guangdong, China	ITS: GQ231968 *trn*H-*psb*A: GQ267064

Table 2. Analysis of the polymorphic bands of ISSR

Primers	Sequences (5'→3')	No. of loci	No. of polymorphic loci	Polymorphism bands (%)
818	CAC ACA CAC ACA CAC AG	11	11	100
819	GTG TGT GTG TGT GTG TA	8	8	100
827	ACA CAC ACA CAC ACA CG	10	8	80
848	CAC ACA CAC ACA CAC ARG	13	12	92.3
855	ACA CAC ACA CAC ACA CYT	8	8	100
885	BHB GAG AGA GAG AGA GA	7	5	71.4
	Total	57	52	91.2

Citrus grandis "Tomentosa"and the common form, with similarity among them at 97.5%[19].

Here, we employ chloroplast *trn*H-*psb*A intergenic spacer, nuclear internal transcribed spacer (ITS) and inter-simple sequence repeats (ISSR) marker to differentiate *C. grandis* 'Tomentosa' and *C. grandis*. Three other common cultivated *Citrus* species, including *C. chachiensis* (*C. reticulata* Blanco var. *chachiensis*), *C. reticulata* Blanco and *C. medica* L. var. *sarcodactylis* (Hoola van Nooten) Swingle, were also studied to prevent the misuse of these valuable herbs.

MATERIALS AND METHODS

I. *Plant Materials*

Fresh samples were collected from various sources, which were identified by Dr. W. B. Liao from Sun Yat-sen University (Guangzhou, China) according to the organoleptic characteristics (Table 1). All samples were washed with double distilled water and rinsed with 70% (v/v) ethanol to remove surface contaminants. Samples were stored in box with silica gel and kept in the School of Life Science, Sun Yat-sen University.

II. *ISSR Studies -DNA Extraction and PCR*

Total DNA for ISSR study was isolated from samples using Dneasy Plant Mini Kit (Qiagen, Germany) according to the instruction of the manufacturer. ISSR amplification reactions were carried out in 25 μL volume containing 20 ng of template DNA, 1× *Taq* buffer [50 mM $(NH_4)_2SO_4$; 75 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.001% gelatin], 1 mM dNTPs, 1 unit of *Taq* polymerase and 1 μM primers (designed by The University of British Columbia). PCR amplification was performed as follows: initial 5 minutes at 94°C, 40 cycles of 30 s at 94°C, 45 s at 55°C, 2 minutes at 72°C, and a final 7 minutes extension at 72°C. PCR amplification products were analyzed on 1.8% (w/v) agarose gel. DNA marker was prepared according to the manufacturer's instruction (Seegene, Korea).

III. *ISSR Data Analysis*

The ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The genetic identity and genetic distance were computed using POPGENE 32, percentage of all loci that were polymorphic regardless of allele frequencies was performed by diploid data analysis of POPGENE 32. A dendrogram was constructed based on Nei's genetic distances using the un-weighted pair-group mean algorithm (UPGMA) of Molecular Evolutionary Genetic Analysis (MEGA) version 4.0[20].

IV. *DNA Sequence Studies - DNA Extraction, PCR and Cloning*

Total DNA was extracted from fresh samples according to a published method[21]. In brief, cetyltrimethyl ammonium bromide method was used for the extraction. Chloroform-isoamyl alcohol (24:1) were added to remove protein, and 2/3 (v/v) isopropanol to precipitate DNA. Finally, DNA pellet was washed with 70% ethanol, and resuspended in water. Primer ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS region[22], while primer psbAF (5'-GTTATGCATGAACGTAATGCTC-3') and trnHR (5'-CGCGCATGGTGGATTCACAAATC-3') were used to amplify *trn*H-*psb*A region[23]. PCR was carried out in a 25 μL mixture containing 10 ng DNA, 1× *Taq* buffer, 1 mM dNTPs, 1 μM primers, and 1 unit of *Taq* polymerase. Samples were initially denatured at 94°C for 5 minutes, and then subjected to 35 PCR cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. PCR products were separated on a 1.5% agarose gel. PCR products were recovered from agarose gel using the Gel-MTM Gel Extraction System (Viogene, Taiwan). Purified DNA fragment was cloned into pGEM-T Easy vector (Promega, USA). Rapid Plasmid Miniprep System (Viogene, Taiwan) was then used for plasmid purification.

V. *DNA Sequencing and Phylogenetic Analysis*

Two colonies for each sample were sequenced. Primer flanking sites on the DNA sequences were removed. DNA sequences were aligned by Clustal W[24,25]. Molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic Analysis (MEGA) version 4.0. Phylogenetic tree was constructed using the maximum parsimony method with default settings. Bootstrap support values were determined using 500 replicates.

RESULTS

I. *ISSR Amplification and Phylogenetic Relationships*

Totally, 76 primers were screened and six of them were capable of generating polymorphic profiles (Figure 1). ISSR primers produced varying numbers of DNA fragments, depending on their SSR motifs. Amplifications using the five 5'-anchored dinucleotide repeat ISSR primers produced an average of 5.1 bands over all the samples, among which, primers based on poly(CA) motif produced seven bands on average.

The six primers based on poly(CA) motif produced 57 bands across 23 samples, of which 52 were polymorphic bands and account for 91.23%. The number of generated bands varied from 7 (ISSR 885) to 13 (ISSR 848),

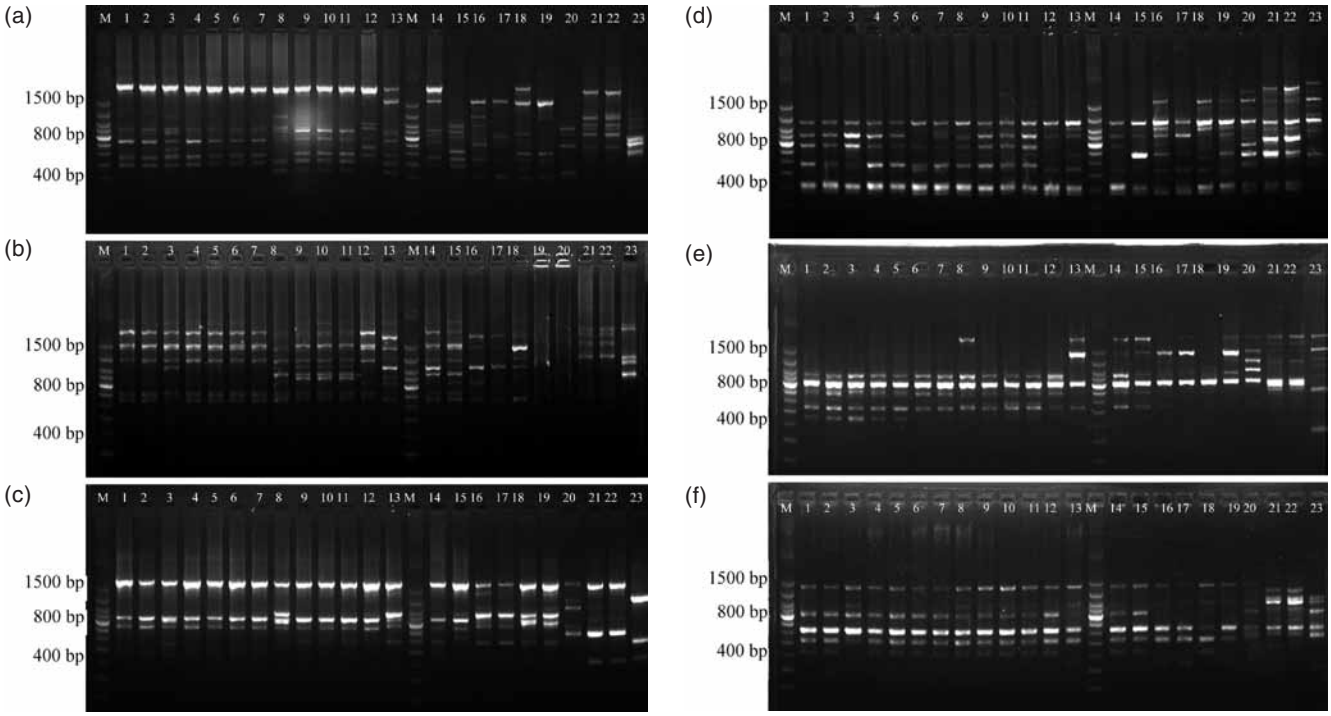

Figure 1. ISSR profiles of the 23 *Citrus* samples using primer (a) 818, (b) 819, (c) 827, (d) 848, (e) 855, (f) 885. M: 100 bp ladder; lane 1-15, HZY01-15; lane 16-19, YO01-04; lane 20, GAN; lane 21-22, JU and lane 23, FO.

and the size ranged from 200 to 2200 bp. The average number of bands and polymorphic bands per primer were 9.5 and 8.7, respectively. Percentage of polymorphism ranged from 71.43% (ISSR 885) to 100% (ISSR 818, 819, 855), with mean 90.62% across all samples. The 3'-anchored primer based on (GA) motifs produced a lower polymorphism rate of 71.43% (Table 2).

The ISSR bands were counted for the presence or absence among samples and the binary scores were used for the UPGMA cluster analysis. The complete data was based on a total of 57 bands. A dendrogram based on UPGMA analysis with ISSR data is shown in Figure 2. The 23 samples were grouped into two clusters. Cluster I is mainly divided into two minor clades, which consist of *C. grandis* "Tomentosa" (HZY) and *C. grandis* Osbeck (YO). These two varieties are closely related and nested in this tree, but form separate clades respectively. Cluster II consist of *C. chachiensis* (GAN), *C. reticulata* (JU) and *C. medica* var. *sarcodactylis* (FO). Contrary to C. *medica* var. *sarcodactylis* (FO), *C. chachiensis* (GAN) and *C. reticulata* (JU) are closely related.

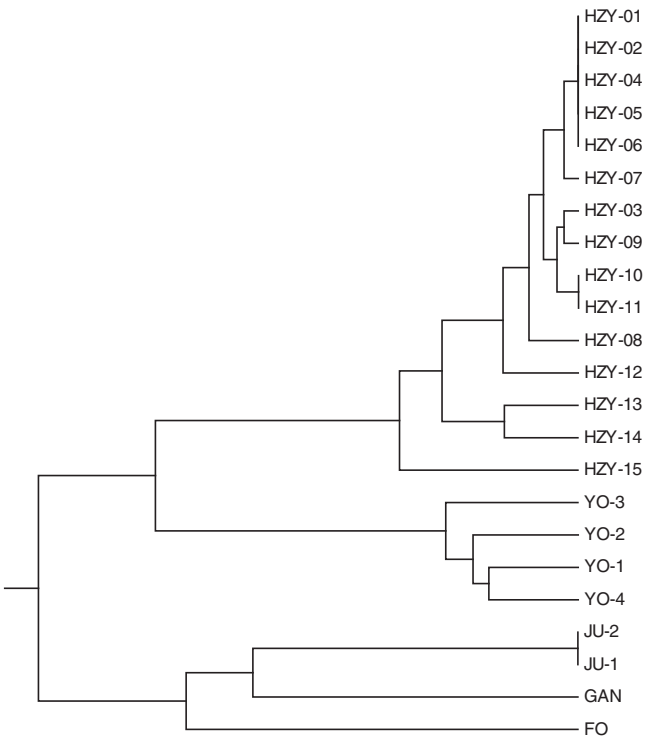

Figure 2. Dendrogram based on the analysis of the ISSR data by UPGMA. Full names of the symbols are listed in Table 1.

II. *Sequence Analysis*

Determined DNA sequences were deposited in GenBank with accession numbers listed in Table 1. Excluding the primer flanking site, the sizes of ITS regions (including partial 18S rRNA, ITS1, 5.8S rRNA, ITS2, and partial 26S rRNA) ranged from 701 bp to 711

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HZY-01      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-08      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-11      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-12      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-13      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-14      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
HZY-15      ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
YO-1        ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
YO-2        ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
YO-3        ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
GAN         ACAGTGCGCTACGGGGTGCAGTGCCTTCTTTCAAATGTATCCAAAACGACTCTCGGCAAT 300
JU          ACGGTGCGCCGCGGGGTGCGGCGCCTTCTTTCACATGTATCCAAAACGACTCTCGGCAAC 300
FO          ACAGTGCGC----------AGCGCCTTCTTTCAAATGTATCCAAAATGACTCTCGGCAAC 290
            ** ******           * ********** ********** **********

HZY-01      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-08      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-11      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-12      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-13      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-14      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
HZY-15      GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
YO-1        GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
YO-2        GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
YO-3        GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
GAN         GGATATCTTAGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATT 360
JU          GGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATT 360
FO          GGATATCTCAGGTCTCGTATCGATGAAGAACATAGCAAAATACGATACTTGGTGTGAATT 350
            ********  * ***** ************ **** **** ******************

HZY-01      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-08      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-11      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-12      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-13      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-14      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
HZY-15      AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
YO-1        AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
YO-2        AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
YO-3        AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAAACCAAGGC 479
GAN         AGGGCATGTCTGCTTGTGTGTCACGCATCGTTGCCCCACCCCACCCCCCGCAACCCAAGGC 479
JU          AGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCTCCACCCCACCCCCCCCAAACCAAGGC 480
FO          AGGGCATGTCTGCCTGTGTGTCATGCATCGTTGCCCCACCCCACCCACGCAAACCAAGGC 470
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Figure 3. ITS sequences of *Citrus grandis* 'Tomentosa' (HZY) and its related species. * denotes nucleotide identical in all sequences. Nucleotides that may be used to differentiate the concerned *Citrus* species are boxed.

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HZY-01          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-08          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-11          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-12          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-13          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-14          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
HZY-15          GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
YO-1            GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGATTGGCC 539
YO-2            GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGATTGGCC 539
YO-3            GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGATTGGCC 539
GAN             GGGGGCCCCAGGGTGCGGGCATAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 539
JU              GGGGGCCCCGGGGTGTGGGCGGAGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCC 540
FO              GGGGGCCCCGGGGTGCGGGCATAGATTGGCCTCCCGTACGCTGACTGCTCGCGGTTGGCC 530
                ********* ***** ****  ************** ******* ******** *****

HZY-01          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
HZY-08          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCATGCAA 658
HZY-11          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
HZY-12          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
HZY-13          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
HZY-14          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
HZY-15          AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
YO-1            AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTATTGACCTTGAAGCTCCACGCAA 658
YO-2            AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTATTGACCTTGAAGCTCCACGCAA 658
YO-3            AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTATTGACCTTGAAGCTCCACGCAA 658
GAN             AGCTACCGCTGCGCGCCCAGTATCCAAGTGTGGACTCTACGACCTTGAAGCTCCACGCAA 658
JU              AGCTCCCGCCACGCGCCCGGTCTCCGAGTGGGGACTCTGCGACCCTGAAGCTCCGCGCAA 660
FO              AGCTCCCGCTGCGCGCCCGATCTCCAAGTGTGGACTCTACGACCCTGAAGCTCCACGCAA 649
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Figure 3. Continued

bp. *C. grandis* "Tomentosa", *C. grandis* Osbeck and *C. chachiensis* were both 710 bp in size. Two polymorphic sites were found between *C. grandis* "Tomentosa"and *C. grandis* Osbeck at position 534 bp and 638 bp (Figure 3). Several characteristic sites in the alignment could help to differentiate between the two *C. grandis variants* and other *Citrus* species. For example, there is a deletion in C. *medica* var. *sarcodactylis* from position 250 to 259 bp.

The sizes of *trn*H-*psb*A region of *C. grandis* 'Tomentosa' samples ranged between 514 and 516 bp. DNA sequences of these samples were slightly shorter than *C. reticulata* (530 bp), but longer than *C. grandis* Osbeck (512-514 bp) and *C. medica var. sarcodactylis* (507 bp), and similar to those of the *C. chachiensis* (516 bp). Alignment showed that this region was conserved between *C. grandis* 'Tomentosa' and *C. grandis* Osbeck as there was only one variable site at position 355 bp. Nevertheless, we could distinguish the two *C. grandis* species from its relative species with the few insertion or deletion sites in the sequences (Figure 4).

Using the determined sequences, ITS and *trn*H-*psb*A phylogenetic trees were constructed. In the ITS tree, *C. grandis* (HZY and YO) and *C. chachiensis* (GAN) were closely related and resolved as a clade with bootstrap value of 100, while the rest *Citrus* species formed another clade (Figure 5). Similar clustering could be found in *trn*H-*psb*A tree: *C. grandis* 'Tomentosa' (HZY), *C. grandis* Osbeck (YO) and *C. chachiensis* (GAN) formed a clade with bootstrap value of 72 (Figure 6). However, sample HZY-15 was clustered with other YO samples to form a subclade, which was different from the ISSR and ITS phylogenetic trees.

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HZY-01      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-08      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-11      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-12      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-13      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-14      GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
HZY-15      GTCTTTGCGTAGGCGGGTTTTTGAAAATAACGGATCAATTCTGACCCCCAGCTGGGGGTC 120
YO-1        GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
YO-2        GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
YO-3        GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
GAN         GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGGCCCCCAGCTGGGGGTC 120
JU          GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAAT-CTGACCCCCAGCTGGGGGTC 119
FO          GTCTTTGTGTAGGCGGGTTTTTGAAAATAACGGATCAATACTGACCCCCAGCTGGGGGTC 120
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HZY-01      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
HZY-08      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 293
HZY-11      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
HZY-12      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
HZY-13      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 291
HZY-14      TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
HZY-15      CGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
YO-1        TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 292
YO-2        TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 289
YO-3        TGTTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 293
GAN         TGCTGGTATGCGCTAATACTACTAATAAATTACTAAATTTCTAAT-------TTTATTAT 293
JU          TGTTGGTATGCGCTAATACTACTAATAAATTAATAAATTTCGAATGTTATTATTTATTAT 298
FO          TGTTGGTATGCGCTAATACTACTAATA--------AATTTCTAAT-------TTTATTAT 276
             * ************************        ****** ***       ********

HZY-08      AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 405
HZY-11      AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 404
HZY-12      AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 404
HZY-13      AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 403
HZY-14      AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 404
HZY-15      AGAAAAAAGCCAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCAGTTGGCTCTTCA 404
YO-1        AGAAAAAAGCCAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 404
YO-2        AGAAAAAAGCCAATAGAAAGGTTGTAGTTTTCTGCTCTTCGACCTTCATTTGGCTCTTCA 401
YO-3        AGAAAAAAGCCAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 405
GAN         AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCTCTTCGATCTTCATTTGGCTCTTCA 405
JU          AGAAAAAAGTCAATAGAAAGGTTGTAGTTTTCTGCCCTTCGATCTTCATTTGGCTCTTCA 418
FO          AGAAAAAAGACAATAGAAAGGTTGTAGTTTTCTGCCCTTCGATTTTCATTTGGTTCTTCA 396
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Figure 4. *trn*H-*psb*A sequences of *Citrus grandis* 'Tomentosa' (HZY) and its related species. * denotes nucleotide identical in all sequences. Nucleotides that may be used to differentiate the concerned *Citrus* species are boxed.

DISCUSSION

ITS region of nuclear ribosomal DNA and chloroplast region *trn*H-*psb*A are often employed to assist identification, particularly at the intergenic level[26-28]. In this study, however, these two DNA regions could not be used to distinguish between *C. grandis* 'Tomentosa' and *C. grandis* Osbeck because there is only slight difference in the DNA sequences. Such high sequence similarity may be due to their close relationship, as also revealed by the ITS and *trn*H-*psb*A sequences of some species from other groups[29,30].

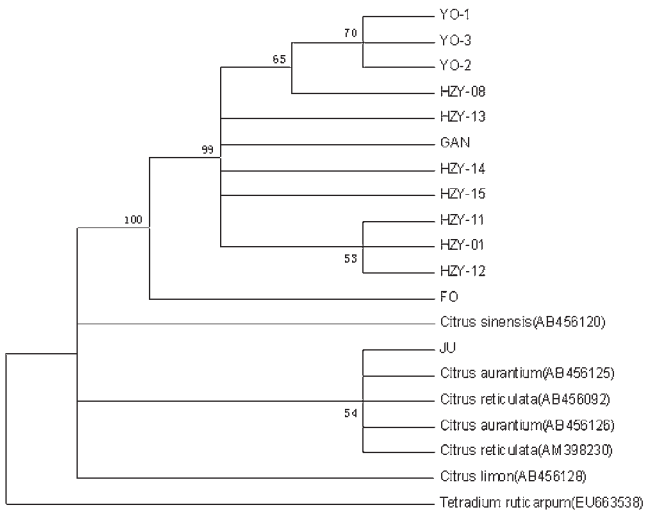

Figure 5. Maximum parsimony tree based on the ITS sequences of *Citrus grandis* 'Tomentosa' (HZY) and its related species. *Tetradium ruticarpum* (EU663538), *C. sinensis* (AB456120), *C. aurantium* (AB456125, AB456126), *C. reticulata* (AB456092, AM398230) and *C. limon* (AB456128) were retrieved from GenBank. *T. ruticarpum* was selected as the outgroup species. Numbers above branches are bootstrap values.

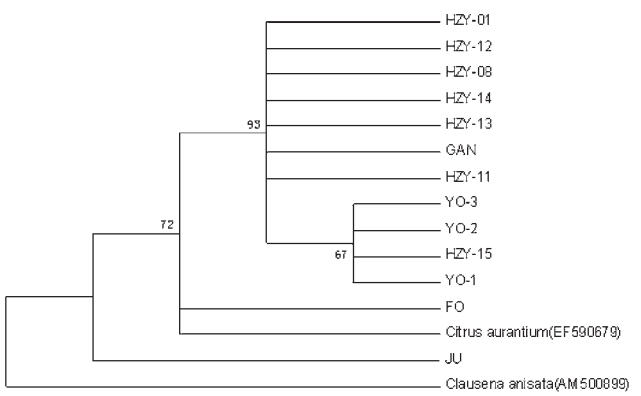

Figure 6. Maximum parsimony tree based on *trn*H-*psb*A sequences of *Citrus grandis* 'Tomentosa' (HZY) and its related species. *Clausena anisata* (AM500899) and *C. aurantium* (EF590679) were retrieved from GenBank, and the former was selected as the outgroup species.

Microsatellite technique samples the whole genome, and is extremely efficient, reproducible and highly informative[10,31-33]. $(AT)_n$ is the most abundant microsatellite in plant nuclear genomes, followed by $(AG)_n$ and $(AC)_n$[10,34]. In our study, primers $(CA)_8G$, $(AC)_8G$, $(CA)_8RG$, $(AC)_8YT$, and $(GT)_8A$ generated unique fingerprints, while primer $(AT)_n$ and $(AG)_n$ produced similar fingerprints between *Citrus grandis* 'Tomentosa' and *C. grandis* Osbeck. We found that the dinucleotide repeats of the primers were easier to produce fingerprint than that of trinuleotide repeats in *Citrus* genomic DNA, probably due to the abundance of the dinucleotide repeats in the *Citrus* genome. This is also consistent with the surveys of microsatellite markers in the sweet orange (*Citrus sinensis* L. Osbeck)[10]. We also found that only $BHB(GA)_7$ generated excellent results in ISSR, while other primers with different number of GA-repeat produced no PCR products. Poor result may be due to either the characteristics of the primers or to the relative abundance of the priming sites in the genome[35].

HZY-15 has less hairs than the other *Citrus grandis* "Tomentosa"samples, and people usually call this type of *C. grandis* Fu Mao Huazhouyou, which means less tomentum on the fruit surface[36]. Instead of clustering with other HZY samples, it forms a subclade with other YO samples in the *trn*H-*psb*A tree. On the other hand, Fu Mao-HZY and HZY cannot be distinguished by the ITS region identification as well as the ISSR UPGMA tree. This indicates that the genetic difference between Fu Mao-HZY and HZY is small.

The most widely accepted taxonomic system for *Citrus* was proposed by Swingle[37] and Tanaka[38]. Subsequent phylogenetic analysis by Barrett and Rhodes suggested that there were only three true species within cultivated *Citrus*, including citron (*Citrus medica* L.), mandarin (*C. reticulata* Blanco) and pomelo (*C. grandis*)[39]. Some cultivated *Citrus* species are used as medicinal materials for centuries in China. According to traditional Chinese medicine, all these *Citrus* variants have therapeutic effects in reducing phlegm and smoothing coughs, but are used to treat different syndromes. The *Citrus* crude drugs could be misused easily when just considering their morphological and chemical characteristics, and thus compromising the pharmaceutical efficacy. In this work, we have shown the DNA sequences in *trn*H-*psb*A and ITS regions for the determination of pomelo and related medicinal material. ISSR fingerprint analysis is able to differentiate between *Citrus grandis* "Tomentosa"from other *Citrus* variants to prevent the misuse of the *Citrus* herbs. Our study also shows that ISSR and DNA sequencing methods are complementary for the differentiation of closely related species in general.

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