The genetic and chemical diversity in three original plants of licorice, *Glycyrriza uralensis* Fisch., *Glycyrrhiza inflata* Bat. and *Glycyrrhiza glabra* L.

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Abstract: Licorice is one of the most frequently used Chinese herbs, mainly containing triterpenoids and flavonoids. Three original plants, *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch., and *Glycyrrhiza inflata* Bat., are defined as licorice in *Chinese pharmacopeia*. In this study, 40 *G. uralensis* samples (Group A), 60 *G. glabra* samples (Group B, C and D) and 40 *G. inflata* samples (Group E and F), were used as plant materials, the genetic diversity of samples were determined by gene sequencing technology and the chemotypic diversity were detected by HPLC. The chemotypic diversity analysis showed that contents of triterpenoids in *G. glabra* (isoglycyrrhizin: 2.483±0.0671 mg·g^{-1}, glycyrrhizin: 34.660±0.8591 mg·g^{-1}) were obviously higher than that in *G. uralensis* and *G. inflata*. However, the contents of flavonoids (liquiritin: 21.996±0.6396 mg·g^{-1}, isoliquiritin: 4.556±0.1252 mg·g^{-1}, liquiritigenin: 0.623±0.0200 mg·g^{-1}, isoliquiritigenin: 0.281±0.008 mg·g^{-1}) in *G. uralensis* were higher than that in *G. glabra* and *G. inflata*. And contents of triterpenoids and flavonoids were both lowest in *G. inflata*. The genetic diversity analysis showed that the *psb*A-*trn*H intergenic regions on chloroplast DNA sequences were same in the same species, and significantly different between any two species. These findings will lay a solid foundation for the identification and quality control of licorice. Furthermore, recently the activity of isoglycyrrhizin has attracted more and more attentions and researches. The HPLC method established in this paper for the simultaneous assay of isoglycyrrhizin and glycyrrhizin will be helpful for the screening of superior quality licorice with a high content of isoglycyrrhizin.

Keywords: Licorice; *Glycyrrhiza uralensis* Fisch.; *Glycyrrhiza inflata* Bat.; *Glycyrrhiza glabra* L.; HPLC; *psb*A-*trn*H.

INTRODUCTION

Glycyrrhiza uralensis Fisch., *Glycyrrhiza inflata* Bat. and *Glycyrrhiza glabra* L. are defined as licorice in *Chinese Pharmacopoeia* (National Pharmacopoeia Committee. 2010). It is one of the most frequently used Chinese herbs for the antitumor (Khan *et al*. 2013; Lin *et al*. 2014), antimicrobial (Long *et al*. 2013; Ahn *et al*. 2012), antiviral (Wang *et al*. 2013; Feng *et al*. 2013), anti-inflammatory (Fu *et al*. 2014; Ishida *et al*. 2013), immunoregulatory (Kim *et al*. 2013; Ma *et al*. 2013), and several other activities (Chakravarthi *et al*. 2013; Chakravarthi *et al*. 2014). Many triterpenoids and flavonoids are responsible for the above activities. Among them, glycyrrhizin (GC) (Bhattacharjee *et al*. 2012), liquiritigenin (LTG) (Gaur *et al*. 2014), isoliquiritigenin (ILTG) (Link *et al*. 2015), liquiritin (LQT) (Teng *et al*. 2014) and isoliquiritin (ILQT) (Wang *et al*. 2008) are the most common active compounds in licorice. However, in recent years, more and more studies have shown that isoglycyrrhizin (IGC) is a better active compound in licorice for higher targeting, better lipotropy, and fewer adverse reactions, and has been used in the treatment of many diseases (Huang *et al*. 2014; Xiao *et al*. 2014; He *et al*. 2010; Chen *et al*. 2014). The studies about content analysis of IGC in licorice were very infrequent.

G. uralensis, *G. inflata* and *G. glabra* are different species with different levels of active compounds. However, in licorice different compounds are responsible for different pharmacological activities. Triterpenoids, such as GC and IGC, are mainly responsible for hepatoprotective and antiviral effects (Matsumoto *et al*. 2013; Wang *et al*. 2012), and flavonoids, such as LQT and ILTG, are mainly responsible for nitrite scavenging capacity and treatment of cancer (Dong *et al*. 2014; Wang *et al*. 2013). The differences in the contents of main active compounds will further influence the pharmacological activities of three original licorice plants. Therefore, revealing the chemotypic diversity in three original plants of licorice are important and meaningful for the reasonable and safe use of licorice.

Traditional morphological identification has always been the main identification method for the three licorice original plants. However, the medicinal parts of licorice are roots and rhizomes, which are very similar in shape (fig. 1). It is very difficult to identify the origin of the

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licorice roots using morphological identification method. Therefore, the establishment of molecular identification method of licorice will be useful for the efficient and accurate identification and reasonable use of licorice. However, the studies about molecular identification of licorice are very limited.

Fig. 1: The licorice cuts from three original plants, *G. uralensis*, *G. glabra*, and *G. inflata*.

In this paper, 6 groups of biennial licorice (one group of *G. uralensis*, three groups of *G. glabra*, and two groups of *G. inflata*) with different morphological characteristics were used as plant materials. The broad objective of this paper was to explore the diversity of genetic and chemotypic features in the three licorice original plants. Depending on our previous studies, *G. uralensis* had a unique internal transcribed spacer (ITS) sequence, which was different from *G. inflata* and *G. glabra*. However, *G. inflata* and *G. glabra* shared same ITS sequence. So ITS sequence can't be used to distinguish the three species. In this paper, we further evaluated the efficiency of *psb*A-*trn*H intergenic region on chloroplast DNA for discriminating the species of licorice. At the same time, the contents of two triterpenoids, GC and IGC, and four flavonoids, LQT, ILQT, LTG and ILTG, were assayed. The results of this paper are intended to facilitate licorice breeding research, assist clinical and pharmacological studies, inform basic phytochemical research, and evaluate the potentials for molecular identification and certification of licorice roots and their products.

MATERIALS AND METHODS

Plant materials

Seeds of *G. uralensis*, *G. inflata* and *G. glabra* were cultured in Tongxin county, Ningxia Hui Autonomous Region, China, for two years. Six groups (Group A: *G. uralensis*, Group B, C, and D: *G. glabra*, Group E and F: *G. inflata*) with different morphological characteristics were collected as samples. The species, number of samples and morphological characteristics of each group are given in table 1. All of the samples were identified by Prof. Chunsheng Liu of Beijing University of Chinese Medicine. Voucher specimens were deposited in the herbarium at the School of Chinese Pharmacy, Beijing University of Chinese Medicine. The leaves of 140 samples were immersed in silica gel during the field collection and used as materials for *psb*A-*trn*H sequence analysis, and the dry roots and rhizomes of 140 samples were used as materials for the content analysis of main compounds.

HPLC-UV analysis

Preparation of samples

The fresh roots of 140 licorice samples were collected and dried to a constant weight. Then 0.1g powder of each sample was extracted with ultrasonic by 50mL 50% methanol for 30 min in 50mL volumetric flask. Samples were filtered by 0.45μm filter membrane before HPLC analysis.

Flavonoids assay

An individual stock solution of standard compounds LQT (purity: 98%), ILQT (purity: 98%), LTG (purity: 98%), and ILTG (purity: 98%) was prepared separately at a concentration of 0.01126 $mg \cdot mL^{-1}$, 0.00448 $mg \cdot mL^{-1}$, 0.001158 $mg \cdot mL^{-1}$ and 0.002272 $mg \cdot mL^{-1}$, in 50% methanol solutions. The calibration curves were prepared at six concentration levels as 1μL, 2μL, 5μL, 10μL, 15μL, and 20μL.

Chromatographic instrument

Agilent 1100 (Agilent Technologies Inc, California, USA). Chromatographic column: CAPCELL PAK C18 MGII (250×4.6 mm, 5 μm, Shiseido Co., LTD, Tokyo, Japan). Detection wavelength: 276 nm (0-13 min), 360 nm (13-23 min), 276 nm (23-28 min), and 376 nm (28-55 nm). Flow rate: 1.0 $mL \cdot min^{-1}$. Column temperature: 30 °C. The gradient elution program is given in table 2, mobile phase A is acetonitrile and mobile phase B is 0.1% phosphoric acid.

The precision was determined with 10 μL stock solutions (n=6), and relative standard deviation (RSD) was calculated. The repeatability was determined with the same test solution (A 04, n=6), and RSD was calculated. The stability was determined with the same test solution (A 04) at different time (0, 2, 4, 6, 8, 10, 12, 24 hours after preparation), and RSD was calculated. The recovery experiment was performed as follows: 0.1g powder of sample A 07 together with 5mL stock solutions were extracted with ultrasonic by 50mL 50% methanol for 30 min in 50 mL volumetric flask, which was used as the test solutions (n=5), and RSD was calculated.

Triterpenoids assay

An individual stock solution of standard compounds IGC (purity: 90.8%) and GC (purity: 98%) was prepared separately at a concentration of 0.010704 $mg \cdot mL^{-1}$ and

0.2164 mg·mL^{-1} in 50% methanol. The calibration curves were prepared at six concentration levels as 1μL, 2μL, 5μL, 10μL, 15μL, and 20μL.

The contents of GC and IGC were analyzed using chromatographic instrument Agilent 1100 (Agilent Technologies Inc, California, USA) and chromatographic column Agela Durashell C18-AM (250 mm×4.6 mm, 5μm, Bonna-Agela Technologies Inc, Tianjin, China) at the column temperature 50°C. The mobile phase consisted of 10mol·L^{-1} ammonium perchlorate (A) and methyl alcohol (B) were applied to the gradient elution of 48% A/52% B. The UV detection wavelength was at 250nm, the flow rate was 0.8mL·min^{-1} and the injection volume was 20μL.

The precision was determined with 10μL stock solution (n=6), and RSD was calculated. The repeatability was determined with same test solution (C 03; n=6), and RSD was calculated. The stability was determined with same test solution (C 03) at different time (0, 2, 4, 6, 8, 10, 12, 24 hours after preparation), and RSD was calculated. The recovery experiment was performed as follows: 0.1 g powder of sample A 07 together with 5 mL stock solutions were extracted with ultrasonic by 50 mL 50% methanol for 30 min in 50 mL volumetric flask, which was used as the test solutions (n=5), and RSD was calculated.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 10.0 statistical package (IBM, USA). The independent-sample *T* test and Pearson's correlation were applied to the analysis.

psbA-trnH analysis

DNA extraction

Total DNA of each sample was extracted from approximately 0.1 g licorice leaves using column plant DNA extraction kit (Beijing Biomed medical technology Co., LTD, Beijing, China), following the manufacturer's instructions. Final DNA concentrations were determined by spectrophotometry and their integrity was examined by electrophoresis in 1% (w/v) agarose gel. DNA of each sample was stored at -20°C and PCR analysis was performed within two days.

PCR amplification and sequencing

The PCR primers were as follows: Forward primer: 5'-GTTATGCATGAACGTAATGCTC-3' and Reverse primer: 5'-CGCGCATGGTGGATTCACAATCC-3'. The cycling parameters of PCR were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 60 sec; a final extension at 72°C for 10 min. The amplified sequences were sequenced in both directions by Shanghai Sangon Biotechnology Co., Ltd.

Sequence analysis

Sequencing peak diagrams were obtained and proofread using Contig Express 9.1 software (Invitrogen Co. USA), and analyzed by the Basic Local Alignment Search Tool (BLAST) of National Center of Biotechnology Information (NCBI). Neighbor-Joining (NJ) trees were constructed using MEGA 5.0 software (Sudhir Kumar and co-authors, USA), based on the Kimura 2-parameter model.

RESULTS

The contents of four flavonoids in 6 groups

In the stability, precision and accuracy tests, the RSD were all less than 3%. In sample recovery experiments, the average recoveries were 99.1% for LQT, 100.4% for ILQT, 98.9% for LTG, and 99.2% for ILTG. Calibration curves of four flavonoids are given in table 3 (X: peak area, Y: content (ug). The calibration curves reflected a good linearity within the tested concentration ranges, which suggested that the developed method was precise and suitable enough for determining LQT, ILQT, LTG, and ILTG in licorice. The retention time of LQT, ILQT, LTG and ILTG were 9.524 min, 20.259 min, 24.781 min, and 35.748 min, respectively. Fig. 2 shows the HPLC chromatograms of four flavonoids in samples of *G. uralensis*, *G. glabra* and *G. inflata*. The contents of LQT, ILQT, LTG, and ILTG in the six groups are showed in fig. 3.

Independent-sample *T* test was applied to analyze the content levels of the four flavonoids in different groups. The independent-sample *T* test results of LQT showed there was no significant difference between group B and D (p=0.133), and there were significant differences between each of the remaining two groups ($p\leq0.05$). The independent-sample *T* test results of ILQT showed there was no significant difference between group B and D (p = 0.121), and group E and F (p=0.064), and there were significant differences between each of the remaining two groups ($p\leq0.05$). The independent-sample *T* test results of LTG showed there was no significant difference between group B and C (p=0.064), group B and D (p=0.622), and group E and F (p=0.076) and the differences between each of the remaining two groups were obvious ($p\leq0.05$). Independent-sample *T* test results of ILTG showed there was no significant difference between group B and C (p= 0.879), group D and E (p=0.222), and group E and F (p = 0.474), and the differences between each of the remaining two groups were also obvious ($p\leq0.05$). These results were in accord with the results of fig. 3. Since group A was *G. uralensis*, group B, C and D were *G. glabra*, and group E and F were *G. inflata*, it can be found that contents of the LQT, ILQT, LTG, and ILTG were consistent among the same species, and varied a lot among different species. It was also demonstrated that the contents of LQT, ILQT, LTG, and ILTG were significantly higher in *G. uralensis* and lower in *G. inflata.*

Table 1: Six sample groups with different morphological characteristics

Groups	Species	Number	The morphological characteristics
A	*G. uralensis*	40	The stem is soft, the top of the leaflet is tip, and the leaf distance is large.
B	*G. glabra*	20	The stem is erect and green, the leaf margin is plicate, and the surface is bright. The number of lobules is less than five.
C	*G. glabra*	20	The stem is erect and green, the leaf margin is plicate, and the surface is bright. The number of lobules is more than five.
D	*G. glabra*	20	The stem is erect and fuchsia, the leaf margin is plicate, and the surface is bright.
E	*G. inflata*	20	The stem is erect and red at base, the leaflet is flat, the leaf is oval and greyish-green.
F	*G. inflata*	20	The stem is erect and fuchsia, the leaflet is flat, and the tender leaf is fuchsia.

Table 2: The gradient elution program for determination of four flavonoids in licorice

Time/min	Mobile phase A/%	Mobile phase B/%
0	20	80
15	25	75
30	40	60
40	65	35
41	95	5
50	95	5
51	20	80
55	20	80

Table 3: Calibration curves data of four flavonoids by HPLC

Compounds	Calibration curves	r^2	Linear range (μg)
LQT	$Y=5\times10^{-7}X+2\times10^{-7}$	1	0.0113~0.2252
ILQT	$Y=3\times10^{-7}X-3\times10^{-8}$	1	4.48×10^{-3}~0.0896
LTG	$Y=3\times10^{-7}X+7\times10^{-8}$	0.998	1.158×10^{-3}~0.2316
ILTG	$Y=2\times10^{-7}X-8\times10^{-8}$	1	2.272×10^{-3}~0.0454

Table 4: Pearson's correlation results according to the contents of flavonoids in six groups.

Group	Species	Pearson correlation coefficients			
		LQT and ILQT	Sig.	LTG and ILTG	Sig.
A	*G.uralensis*	0.893	0.000	0.806	0.000
B	*G. glabra*	0.966	0.000	0.825	0.000
C	*G. glabra*	0.975	0.000	0.844	0.000
D	*G. glabra*	0.967	0.000	0.953	0.000
E	*G. inflata*	0.943	0.000	0.870	0.000
F	*G. inflata*	0.934	0.000	0.871	0.000

Table 5: Calibration curves data of IGC and GC by HPLC

Compounds	Calibration curves	r^2	Linear range (μg)
IGC	$Y=9\times10^{-7}X+8\times10^{-7}$	0.9999	0.0107~0.2141
GC	$Y=1\times10^{-6}X-1\times10^{-6}$	1	0.2164~4.3280

Table 6: Pearson's correlation results according to the contents of triterpenoids in six groups.

Group	Species	Pearson correlation coefficients	
		GC and IGC	Sig.
A	*G. uralensis*	0.875	0.000
B	*G. glabra*	0.258	0.247
C	*G. glabra*	0.725	0.000
D	*G. glabra*	0.930	0.000
E	*G. inflata*	0.947	0.000
F	*G. inflata*	0.909	0.000

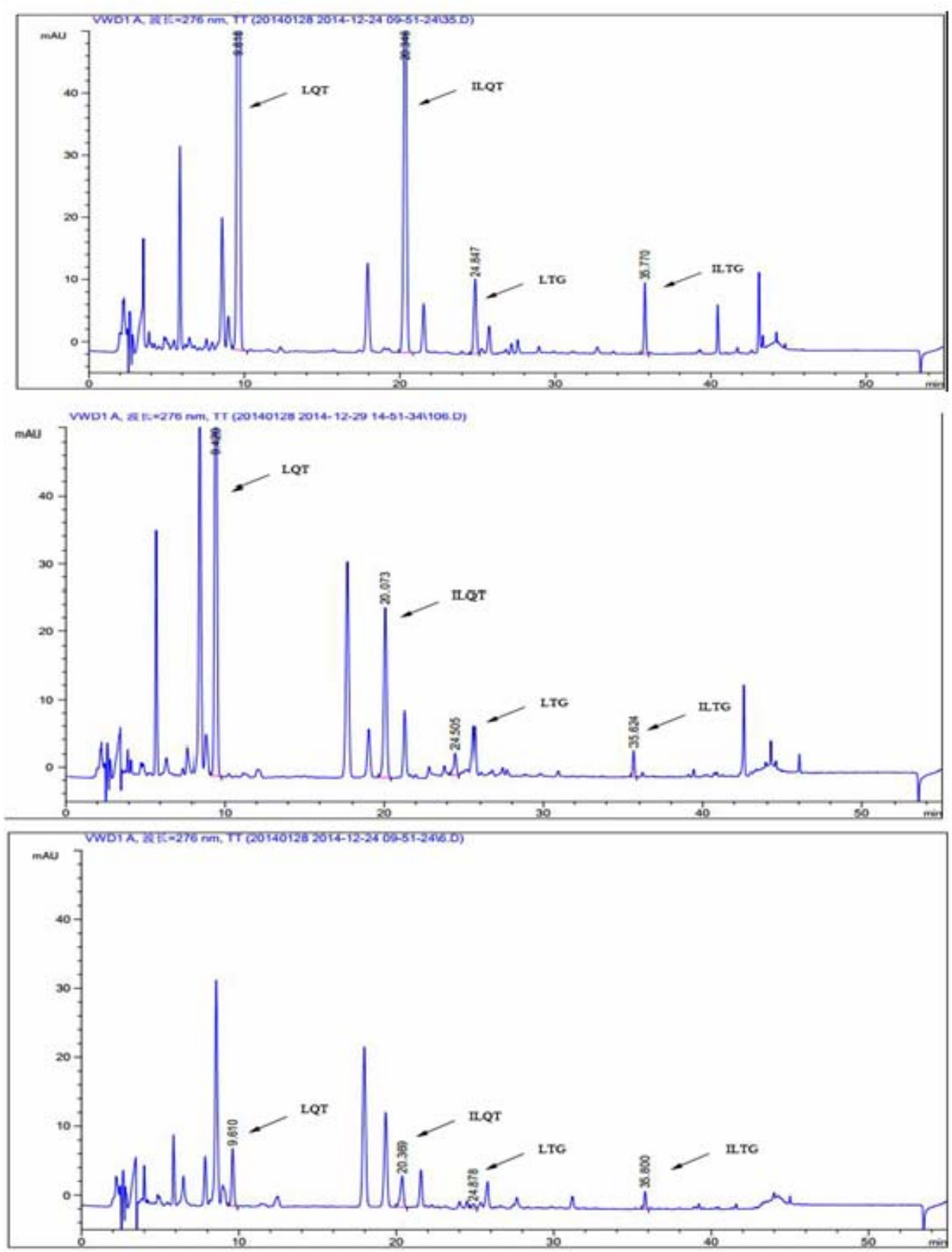

Fig. 2: The HPLC chromatograms of four flavonoids in *G. uralensis* (A: Sample No. A06), *G. glabra* (B: Sample No. C06) and *G.inflata* (C: Sample No. E06)

Table 7: Haplotypes present in *psb*A-*trn*H sequences from *G. uralensis*, *G. glabra*, and *G. inflata*.

Groups	Species	Haplotypes	*psb*A-*trn*H variable sites (bp)		
			224	270	323
A	*G.uralensis*	A	A	C	G
B	*G. glabra*	B	A	T	A
C	*G. glabra*	B	A	T	A
D	*G. glabra*	B	A	T	A
E	*G. inflata*	C	C	T	G
F	*G. inflata*	C	C	T	G

Furthermore, depending on the results of Pearson's correlation, interestingly, it was found that the content of LQT was strikingly correlated to the content of ILQT. What's more, the content of LTG was also significantly correlated with ILTG in all six groups. The results of Pearson correlation coefficients are given in table 4.

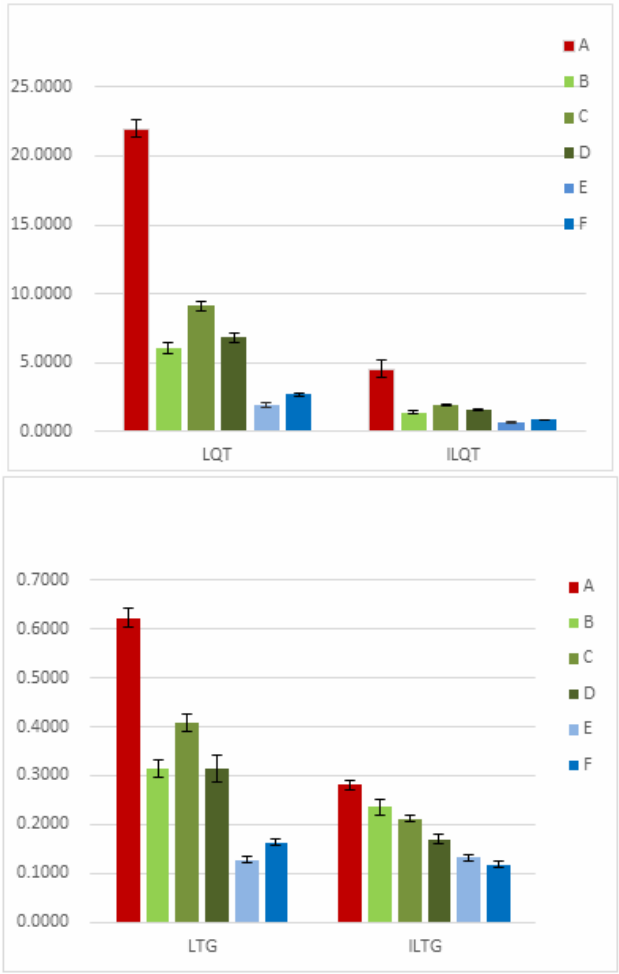

Fig. 3: The contents of four flavonoids in six groups. A**:** The contents of LQT and ILQT in *G.uralensis* (Group A, marked in red), *G. glabra* (Group B, C, and D, marked in green), and *G. inflata* (Group E and F, marked in blue). B: The contents of LTG and ILTG in *G.uralensis* (Group A, marked in red), *G. glabra* (Group B, C, and D, marked in green), and *G. inflata* (Group E and F, marked in blue)

The contents of two triterpenoids in six groups

In the stability test, the precision test and the accuracy test, the RSD of peak areas were all less than 3%. In sample recovery experiments, the average recoveries were 94.1% for IGC, and 100.6% for GC. Calibration curves for the quantitation of two triterpenoids were generated by plotting relationship between the peak area (X) and content (μg) of the standard solution. The data given in table 5 declared that the calibration curves reflect a good linearity within the tested concentration ranges, which suggested that the developed method was precise and suitable enough for determining IGC and GC in licorice.

The retention time of IGC was 12.537 min, and the retention time of GC was 13.546 min. fig. 4 shows the HPLC chromatograms of two triterpenoids in samples of *G. uralensis*, *G. glabra*, and *G. inflata*. The contents of GC and IGC in the six groups are showed in fig. 5.

Independent-sample *T* test was applied to analyze the content levels of the two triterpenoids in different groups. The independent-sample *T* test results of IGC showed there was no significant difference between group A and D ($p = 0.108$), group B and C ($p = 0.313$), group C and D ($p = 0.133$), group B and D ($p = 0.398$), and group E and F ($p = 0.725$), and there were significant differences between each of the remaining two groups ($p \leq 0.05$). The independent-sample *T* test results of GC showed there was no significant difference between group A and D ($p = 0.851$), group B and C ($p = 0.963$), and group E and F ($p = 0.526$), and the differences between each of the remaining two groups were rather obvious ($p \leq 0.05$). These results are in accord with the results in fig. 5. Since group A was *G. uralensis*, group B, C and D were *G. glabra*, and group E and F were *G. inflata*. Considering each species as independent sample, it has been found that there were significant differences between the three original plants. It was also demonstrated that the contents of IGC and GC were significantly higher in *G. glabra* and lower in *G. inflata.*

Furthermore, depending on the results of Pearson's correlation, it was found that the content of IGC was strikingly correlated to the content of GC except group B. The results of Pearson correlation coefficients are given in table 6.

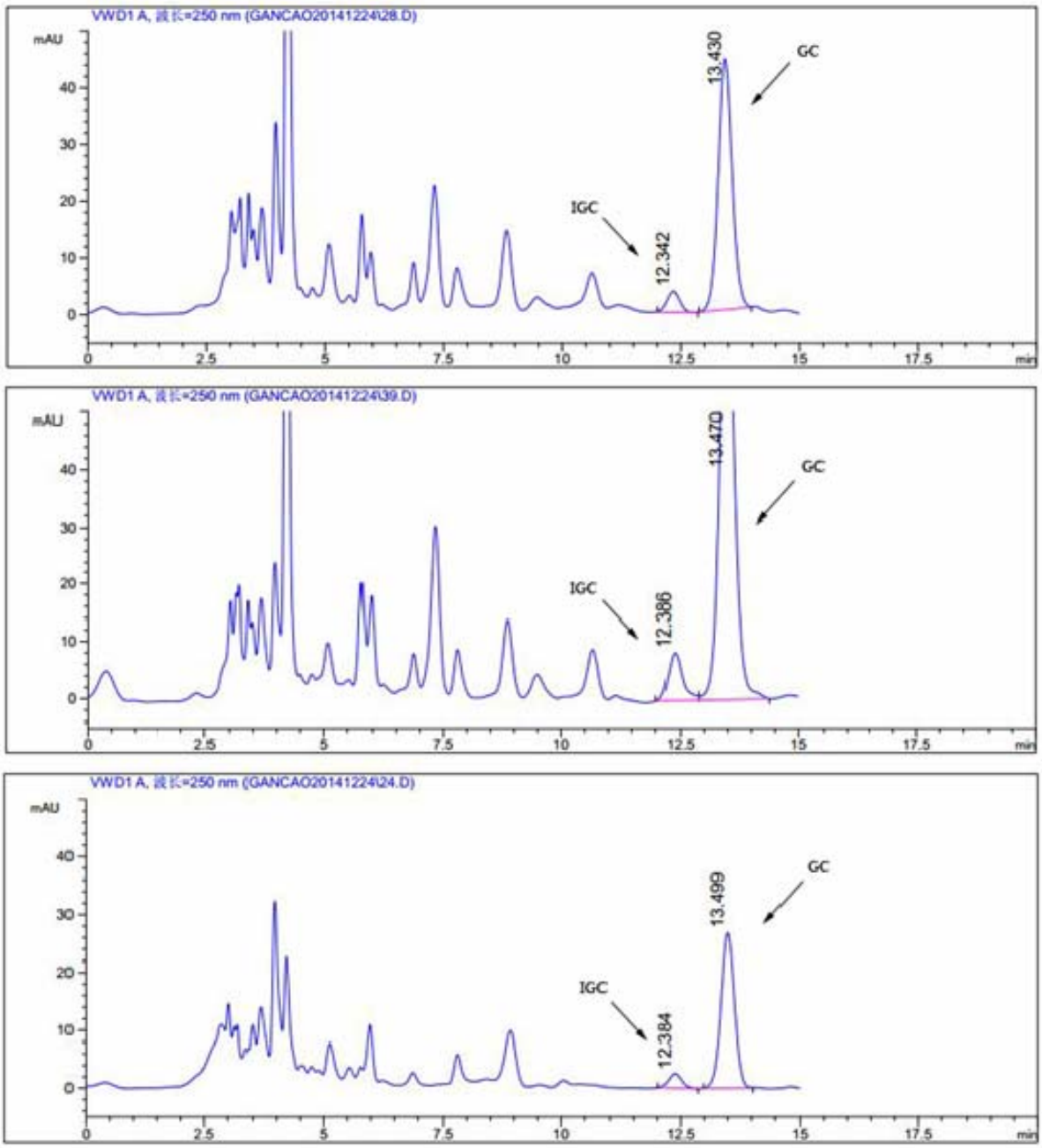

Fig. 4: The HPLC chromatograms of IGC and GC in *G. uralensis* (A, Sample No. A06), *G. glabra* (B, Sample No. C06), and *G.inflata* (C, Sample No. E06).

psbA-trnH sequences analysis results

As shown in fig. 6, *psb*A-*trn*H sequences with a correct length of 389 bp were obtained by PCR. And the sequencing results confirmed that these *psb*A-*trn*H sequences were correct. According to the sequencing results of 140 samples, three variable sites, 224 bp, 270 bp and 323bp were discovered, and three haplotypes were determined as follows: type A had an adenine (A) at 224 bp site, a cytosine (C) at 270 bp site, and a guanine (G) at 323 bp site, type B had an A at 224 bp site, a T at 270 bp site, and an A at 323 bp site, type C had a C at 224 bp site, a T at 270 bp site, and a G at 323 bp site (table 7). It was found that all of the *G. uralensis* samples belonged to type A, all of the *G. glabra* samples belonged to type B, and all of the *G. inflata* samples belonged to type C.

Fig. 7 shows the NJ tree based on 140 *psb*A-*trn*H sequences. It was according with the above results. Three haplotypes, type A, B and C, were well separated. Three original plants of licorice can be easily distinguished using *psb*A-*trn*H sequences.

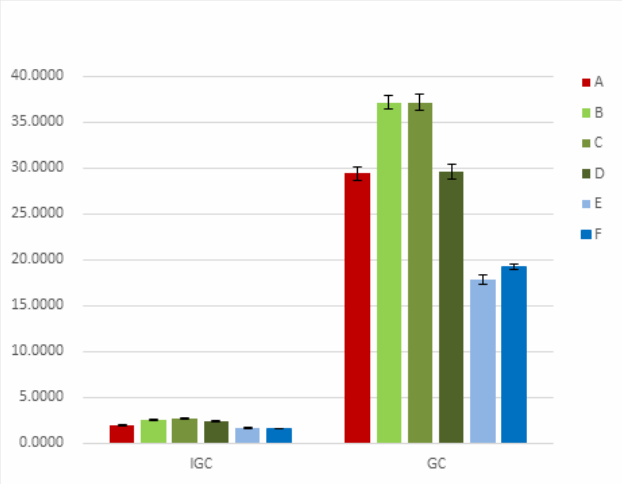

Fig. 5: The contents of IGC and GC in *G.uralensis* (Group A, marked in red), *G. glabra* (Group B, C, and D, marked in green), and *G. inflata* (Group E and F, marked in blue).

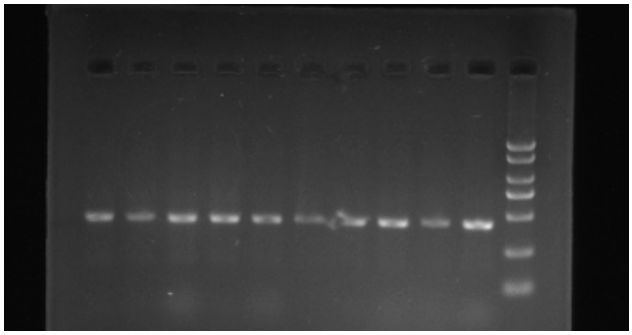

Fig. 6: PCR analysis results of *psb*A-*trn*H sequences (No. 1-3 are the *psb*A-*trn*H sequences from *G. uralensis*, No. 4-6 are the *psb*A-*trn*H sequences from *G. glabra*, No. 7-10 are the *psb*A-*trn*H sequences from *G. inflata*).

DISCUSSION

Different compounds play different roles for the treatment of diseases. According to the chemical features of the investigated licorice, the contents of flavonoids including LQT, ILQT, LTG, and ILTG were much higher in *G. uralensis* compared to *G. glabra and G. inflata.* Therefore, *G. uralensis* will be a better choice for nitrite scavenging capacity and the treatment of cancer. Furthermore, the contents of triterpenoids including IGC and GC were extremely higher in *G. glabra* compared to *G. uralensis* and *G. inflata*. Therefore, it can be regarded as a preferable source of triterpenoids for the superior hepatoprotective effects and antiviral effects. However, in *G. inflata*, whether contents of triterpenoids or contents of flavonoids were significantly lower than the other two species, which demonstrated that it was not a good resource of licorice. And in fact, the actual use of *G. inflata* is rather less than the other two species in Chinese medicine.

Depending on the correlationship analysis results, the content of LQT and ILQT, LTG and ILTG, GC and IGC were strikingly related, since they were three pairs of epimers, there must be some relationships between each other. In addition, since the species which had a higher content of triterpenoids performed poorer in the content of flavonoids there must be some relationships between the biosynthetic pathway of triterpenoids and flavonoids in licorice, which is worth further studying.

In 2009, the Consortium for the Barcode of Life plant working group recommended the combination of *rbc*L+*mat*K as the plant barcode, since they could discriminate among 907 samples from 550 species at the species level with a probability of 72% (CBOL Plant Working Group, 2009). In 2010, with the researches deepen, Chen *et al* (Chen *et al*, 2010) compared seven candidate DNA barcodes (*psb*A-*trn*H, *mat*K, *rbc*L, *rpo*C1, *ycf*5, ITS2, and ITS) from medicinal plant species, they tested the discrimination ability of ITS2 in more than 6600 plant samples belonging to 4800 species from 753 distinct genera and found that the rate of successful identification with the ITS2 was 92.7% at the species level. In 2012, they analyzed the ability of *psb*A-*trn*H and its combinations to discriminate species, and found that the identification rates of *psb*A-*trn*H+ITS2 were significantly higher than those of *mat*K+*rbc*L in 18 families and 21 genera, which suggested that *psb*A-*trn*H +ITS2 combination perform better or equally well compared with other combinations (Pang *et al*, 2012). In the subsequent researches, the molecular identification of medicinal plants has attracted more and more attentions. It has been used in the identification of *Curcuma longa* L. (Parvathy *et al*. 2015), *Rhodiola Genus* (Zhang *et al*. 2015) and many other medicinal plants (Zhou *et al*., 2014; Zheng *et al*., 2014). Among numerous DNA barcodes, ITS sequence and *psb*A-*trn*H intergenic region sequence were believed to be of higher identification efficiency than other sequences (Chao *et al*., 2014; Liu *et al*., 2014; Huang *et al*., 2015). In our study, *G. uralensis* had a unique ITS sequence, which was different from *G. inflata* and *G. glabra*. Three species of licorice were well separated according to three variable sites in *psb*A-*trn*H sequences. So, agreed with former published reports about DNA barcodes, we assumed that ITS and *psb*A-*trn*H could be the suitable genetic markers and their combination could be optical for the species identification of licorice. Since there have been some reports about the different DNA molecular markers such as RAPD (Xin *et al*., 2014), ISSR (Ding *et al*. 2013), and SRAP (Song *et al*., 2010) for the estimation of genetic diversity in plant populations, they also worthy of our further study. We hope this work is helpful for the researches involved in high-quality licorice breeding, safe and effective use of licorice, basic phytochemical analysis, and molecular identification of licorice roots and their products.

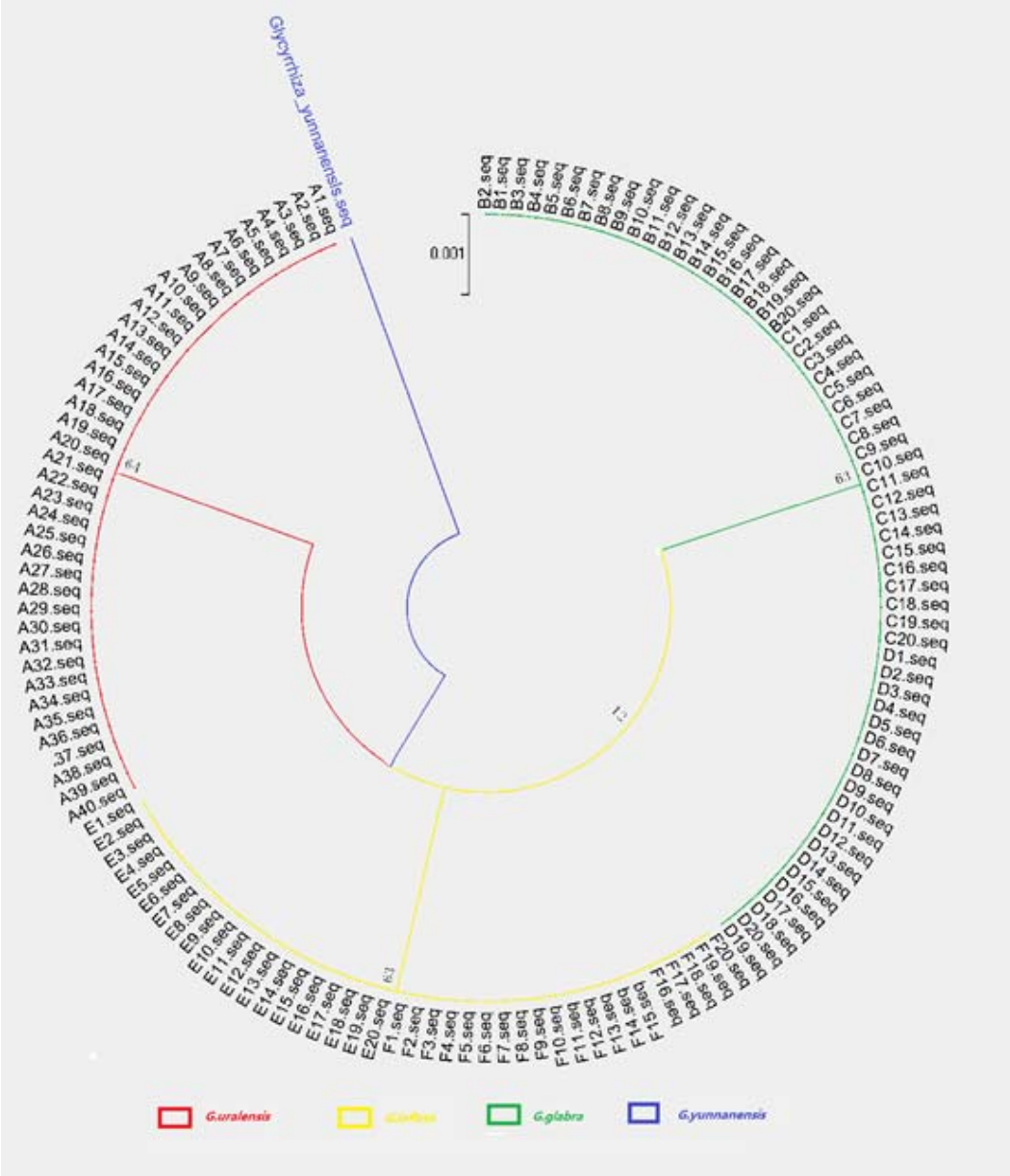

Fig. 7: NJ tree based on140 *psb*A-*trn*H sequences.

CONCLUSION

In this study, in order to simultaneously investigate the contents of different triterpenoids in licorice, a new HPLC method was established, the chemical characteristics and *psb*A-*trn*H haplotypes of six licorice groups (group A: *G. uralensis*, group B, C, and D: *G. glabra*, group E and F: *G. inflata*) with different morphological characteristics were investigated. It has been found that same licorice species shared the same chemical and genetic features, while characteristics varied a lot between each other. The *psb*A-*trn*H sequences of 40 *G. uralensis* samples were all type A (224A-270C-323G), and the contents of flavonoids were obviously higher than that in the other two species. The *psb*A-*trn*H sequences of 60 *G. glabra* samples were all type B (224A-270T-323A), and the contents of triterpenoids were obviously higher than that in the other two species. The *psb*A-*trn*H sequences of 40 *G. inflata* samples were all type C (224C-270T-323G), and both of the flavonoids and triterpenoids contents were the lowest. What's more, in all of the six groups, the content of LQT was strikingly correlated to the content of ILQT, the content of LTG was significantly correlated with ILTG, and the content of IGC was correlated to the content of GC obviously.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation of China (81503181).

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