Identification of *Cistanche* Species by Chemical and Inter-simple Sequence Repeat Fingerprinting

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Received April 28, 2008; accepted October 30, 2008; published online November 4, 2008

Herba Cistanches is a common traditional Chinese medicine that has been used to reinforce the vital function of kidney and induce laxation for more than two thousands years. Four *Cistanche* species were found as Herba Cistanches in China herbal markets, including *C. deserticola*, *C. tubulosa*, *C. salsa* and *C. sinensis*. Phenylethanoid glycosides, particularly echinacoside and acteoside, are considered as active ingredients in *Cistanche* species. The contents of these compounds showed variation in different species and geographical sources. Standard chemical fingerprints were generated from each of four *Cistanche* species, which could be identification markers. In genetic analysis of *Cistanche* species, ninety-four inter-simple sequence repeat (ISSR) primers were used for polymerase chain reaction (PCR) amplification, and of which eight primers were found to be sufficient to distinguish different *Cistanche* species. As a result, the chemical fingerprint combined with the genetic fingerprint for distinction of *Cistanche* species could serve as markers for quality control of Herba Cistanches.

Key words authentic identification; *Cistanche*; fingerprint; HPLC; inter-simple sequence repeat

Herba Cistanches, the stems of *Cistanche* species (Orobanchaceae), is a common traditional Chinese medicine with a reputable name as "Desert Ginseng". Herba Cistanches was first recorded in *Shen Nong Bencao Jing* in *ca.* 100 B.C. as a top grade and used to treat various diseases including impotence, female infertility, morbid leukorrhea, profuse metrorrhagia, cold sensation in the loins and knees, and chronic constipation in the aged.[1)] Nowadays, it is reported to enhance sex and learning behavior,[2)] and to possess the sedative and vasorelaxant effects.[3,4)] Besides its common uses in China, Herba Cistanches is also used as a healthy food supplement for men's care in Japan and Southeast Asia. Therefore, the demand for Herba Cistanches is increasing. Four *Cistanche* species occur in Northwest China, including *C. deserticola* Y. C. MA, *C. tubulosa* (SCHRENK) WIGHT, *C. salsa* (C. A. MEY.) G. BECK and *C. sinensis* G. BECK. The Chinese Pharmacopoeia (2005 edition) recorded that Herba Cistanches should be the dried fleshy stems of *C. deserticola* and *C. tubulosa*. However, *C. salsa* and *C. sinensis* were found in the local markets in China, which could serve as substitutes of Herba Cistanches due to the shortage of *C. deserticola* and *C. tubulosa*.[5)]

Although the aforementioned four *Cistanche* species belong to the same genus, the chemical compositions of these plants were different.[6,7)] Phenylethanoid glycosides are main chemical ingredients in *Cistanche* species, and their amounts showed variation among different species.[8)] The ingredients associated with the pharmacological activities of Herba Cistanches are echinacoside and acteoside, which have significant vasorelaxant, neuroprotective, hepatoprotective effects.[4,9—11)] The levels of these two constituents are commonly used as quality control markers of Herba Cistanches.

Identification of the specific *Cistanche* species is difficult if it was only based on the pharmacognostical characteristics. To ensure the quality of Herba Cistanches, several approaches have been applied for the differentiation of *Cistanche* species. Tomari *et al.* has distinguished *C. deserticola*, *C. salsa* and *C. tubulosa* by comparing the nucleotide sequences of the plastid *rps2* gene and the intergenic spacer region between *rpl16* and *rpl14*.[12)] Jiang *et al.* has developed a HPLC- Diode Array Detector (DAD)-MS fingerprint method to comparatively analyze *Cistanche* species.[13)]

In this study, we determined the chemical and genetic distinctions of stems derived from *C. deserticola*, *C. tubulosa*, *C. salsa* and *C. sinensis*. Chemically, we developed a simple and valid fingerprinting method by HPLC-UV to distinct four *Cistanche* species, and the level of echinacoside and acteoside in different species was also presented. Genetically, inter-simple sequence repeat (ISSR) fingerprint was used to identify different species of *Cistanche*.

MATERIALS AND METHODS

Plant Materials A total of 32 individuals were sampled from 3 sites throughout Northwest of China, which are primary distribution areas of *Cistanche* plants, included Xinjiang, Neimenggu, and Ningxia Autonomous Region (Table 1). They were collected in different date and area, and identified by one of us (Prof. Pengfei Tu). Voucher specimens were deposited at School of Pharmacy, Shanghai Jiaotong University. Each plant sample was cut to two parts from length wise. One part was stored at −20 °C until DNA extraction; the other part was cut to *ca.* 4 mm slides, then put in 70 °C water to kill enzymes, dried in cool for HPLC analysis.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification Genomic DNA of samples was isolated from their fresh tissues using an extraction protocol by DNeasy Plant mini Kit (QIAGEN Inc.). Purified DNA was adjusted to a final concentration of 20 ng/μl for use. PCR amplification was performed in a total volume of 25 μl reaction solution. Each reaction was composed of 60 ng of genomic DNA, 1×PCR buffer, 2 mM $MgCl_2$, 200 μM dNTP, 0.2 μM primer and 1 unit *Taq* DNA polymerase (Sangon Inc., Shanghai, China). Amplification was carried in a PTC-100 thermocycler (MJ Research Inc.) under the following conditions: a hot start step of 5 min at 94 °C, followed by 10 cycles of 50 s at 94 °C, 1 min at 59 °C, 1 min at 72 °C using a touchdown program with 0.5 °C annealing temperature decrements

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Table 1. Contents of Echinacoside and Acteoside from Different Species of Herba Cistanches

Species	Geographical origins	Harvesting time	Content (mg/g)	
			Echinacoside	Acteoside
C. deserticola (Cde-1)	Jimusaer, Xinjiang	April 2005	1.1	4.8
C. deserticola (Cde-2)	Yongning, Ningxia	June 2003	1.7	6.4
C. deserticola (Cde-3)	Alashan, Neimenggu	June 2003	1.5	6.2
C. deserticola (Cde-4)	Alashan, Neimenggu	June 2003	1.7	9.8
C. deserticola (Cde-5)	Guejina, Neimenggu	May 2004	1.9	6.4
C. deserticola (Cde-6)	Alashan left, Neimenggu	May 2004	1.4	5.8
C. deserticola (Cde-7)	Alashan left, Neimenggu	May 2004	1.5	7.0
C. deserticola (Cde-8)	Alashan right, Neimenggu	May 2004	2.2	6.8
C. deserticola (Cde-9)	Alashan right, Neimenggu	May 2004	2.0	15.2
C. deserticola (Cde-10)	Alashan right, Neimenggu	May 2004	2.3	11.6
C. tubulosa (Ctu-1)	Yutian, Xinjiang	November 2002	31.8	10.4
C. tubulosa (Ctu-2)	Yutian, Xinjiang	November 2002	60.3	8.6
C. tubulosa (Ctu-3)	Yutian, Xinjiang	November 2002	65.2	22.1
C. tubulosa (Ctu-4)	Yutian, Xinjiang	November 2002	48.1	8.6
C. tubulosa (Ctu-5)	Yutian, Xinjiang	November 2002	85.4	22.1
C. tubulosa (Ctu-6)	Yutian, Xinjiang	May 2004	45.2	15.9
C. tubulosa (Ctu-7)	Yutian, Xinjiang	November 2002	46.8	7.3
C. tubulosa (Ctu-8)	Yutian, Xinjiang	November 2002	51.9	13.9
C. tubulosa (Ctu-9)	Yutian, Xinjiang	November 2002	119.9	25.4
C. tubulosa (Ctu-10)	Yutian, Xinjiang	May 2004	45.7	14.3
C. salsa (Csa-1)	Yanchi, Ningxia	May 2004	4.7	5.5
C. salsa (Csa-2)	Yanchi, Ningxia	May 2004	4.8	6.7
C. salsa (Csa-3)	Yanchi, Ningxia	May 2004	4.2	6.3
C. salsa (Csa-4)	Yanchi, Ningxia	May 2004	5.2	6.7
C. salsa (Csa-5)	Yanchi, Ningxia	June 2003	4.4	7.9
C. salsa (Csa-6)	Yanchi, Ningxia	June 2003	6.8	8.2
C. sinensis (Csi-1)	Yanchi, Ningxia	May 2003	—	5.3
C. sinensis (Csi-2)	Yanchi, Ningxia	May 2003	—	6.5
C. sinensis (Csi-3)	Yanchi, Ningxia	May 2004	—	8.4
C. sinensis (Csi-4)	Alashan left, Neimenggu	June 2003	—	4.5
C. sinensis (Csi-5)	Alashan left, Neimenggu	June 2003	—	5.2
C. sinensis (Csi-6)	Alashan left, Neimenggu	June 2003	—	4.0

per cycle until 54 °C was attained, followed by 30 cycles of 94 °C for 50 s, 54 °C for 1 min, 72 °C for 1 min and then a terminal extension at 72 °C for 8 min, followed by cooling at 4 °C to stop the reaction. The PCR products were stored at −20 °C until electrophoresis. The genomic DNA extracts were run in 1% agarose gels, PCR products were electrophoresed in a mixed 2% agarose, 0.5 μg/ml EB, photographed using a BIODoc-It^{TM} System (UVP Inc.). Gene Ruler 1 kb DNA ladder plus (Sangon Inc., Shanghai, China) was used as a marker to determine the size of the ISSR fragments. All these conditions used throughout the study for each primer were identical.

Data Analysis The amplification products were scored in terms of a binary code as present (1) or absent (0), each of which was treated as a unit character regardless of its intensity. A genetic identity matrix, using the Nei & Li's coefficient, was constructed using the computer program PopGen v.1.31. Phenetic dedrogram, based on the genetic identity matrix comparing individuals, were constructed using the unweighted pair group method of arithmetic averages (UPGMA, PopGen v.1.31).

Sample Preparation and HPLC Analysis Before use, the materials were dried to constant weight in an oven at 50 °C (Yihen, Shanghai, China) then pulverized and sieved (60 meshs). A 1.0-g powder of sample was extracted with 30.0 ml methanol in an ultrasonic water bath for 30 min. The extracted solution was centrifuged at 12000 rpm for 3 min, the supernatant was filtrated through a 0.2 μm membrane filter before injection. Each sample was prepared with the above protocol for HPLC analysis. HPLC analysis was performed on a Shimadzu LC 2010A liquid chromatograph system (Shimadzu Co., Japan) consisting of a quaternary pump, a column oven, an autosampler. Samples were separated on a Shim-pack VP-ODS C_{18} (4.6 mm i.d.×250 mm, 5 μm, Shimadzu Co., Japan). The mobile phase consisted of 0.095% phosphoric acid in deionized water (A) and 0.095% phosphoric acid in acetonitrile (B) using gradient program of 0—12% (B) in 0—8 min, 12—16% (B) in 8—20 min, 16—20% (B) in 20—35 min, 20—25% (B) in 35—55 min, 25—30% (B) in 55—70 min, 30—0% (B) in 70—90 min. The flow rate was 1.0 ml/min, the injection volume was 10 μl and column temperature was maintained at 25 °C. UV detector was set at 334 nm for acquiring chromatograms.

RESULTS AND DISCUSSION

To develop a fingerprint for Herba Cistanches, an optimized strategy for HPLC conditions was performed. Analysis with methanol had longer duration than those with acetonitrile. The acetonitrile–water system had the same analytical time as the acid acetonitrile–acid water system, but the former had a poor resolution. So the 0.095% phosphoric acid in acetonitrile–0.095% phosphoric acid in water system was chosen for its well baseline resolution and suitable duration for analysis. In order to obtain a sufficiently large number of detectable peaks on the HPLC chromatogram, the UV spec-

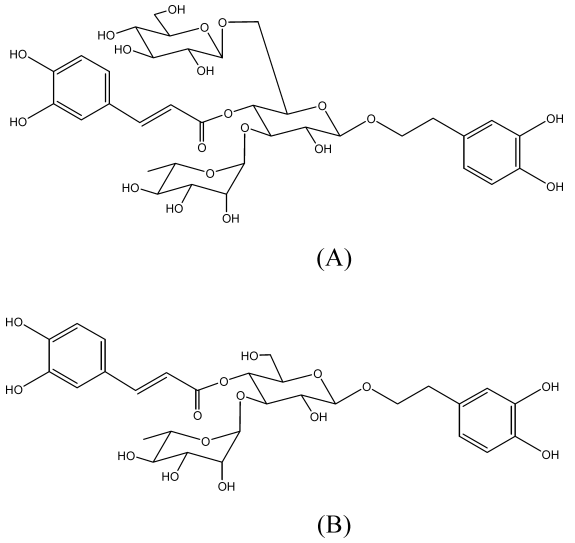

Fig. 1. Structures of Echinacoside (A) and Acteoside (B)

tra of samples were investigated and 334 nm was selected as detection wavelength.

The major constituents of Herba Cistanches are echinacoside and acteoside, which could be determined by HPLC analysis (Fig. 2). The HPLC calibration curves of echinacoside and acteoside exhibited good linearity in a range from 1 to 22 μg. The correlation coefficients of these two compounds were 0.9999. The precision test within the same sample of chemical markers (n=6) showed the relative standard deviation (RSD) values of 0.92% (echinacoside), 0.93% (acteoside). The short-term (12 h) and long-term (24 h) repeatabilities of echinacoside and acteoside were calculated for six determinations (n=6). The RSD of short (or long) term repeatability for echinacoside and acteoside was 1.35% (1.24%) and 1.82% (1.19%), respectively. The recovery experiment was carried out to evaluate the accuracy of the method. Known amounts of echinacoside and acteoside were added to the herbal sample and were extracted by using the ultrasonication method accordingly; the extracted material was subjected to analysis, and the contents of these compounds were calibrated. The average recoveries of the tested chemicals were 96.9% (echinacoside) and 96.4% (acteoside) (n=9). The contents of the two compounds in the samples were quantified and the results were shown in Table 1 with the mean values of three replicate injections. *C. tubulosa* contained much more 12-fold amount of echinacoside and 2-fold amount of acteoside when compared with other three *Cistanche* species. Meanwhile, the content of two constituents is some different in various batches of *Cistanche* species, which is due to the variation of habitat, climate, circumstances, collecting time and soil condition.

In the present study, 10 batches *C. deserticola*, 10 batches *C. tubulosa*, 6 batches *C. salsa*, 6 batches *C. sinensis* were analyzed to evaluate similarities, respectively. The correlation coefficients obtained are listed in Table 2. The results indicated that the similarities of *C. deserticola* were relatively low, which means the secondary metabolites of *C. deserticola* would vary in different locations. *C. tubulosa* were generally consistent except two samples showed some differences. The results of *C. salsa* samples were found resembling

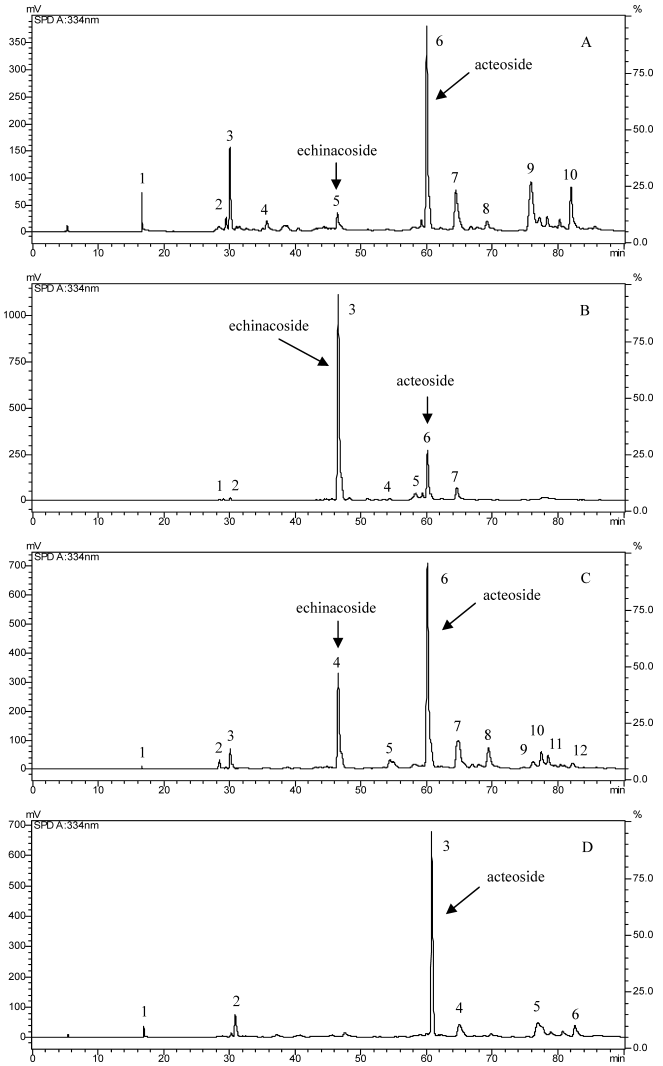

Fig. 2. Standard Fingerprint of *Cistanche* Species: *C. deserticola* (A), *C. tubulosa* (B), *C. salsa* (C), *C. sinensis* (D)

Table 2. The Similarities of HPLC Fingerprint from Different *Cistanche* Species

No.	Similarities	No.	Similarities	No.	Similarities	No.	Similarities
Cde-1	0.775	Ctu-1	0.989	Csa-1	0.952	Csi-1	0.959
Cde-2	0.843	Ctu-2	0.982	Csa-2	0.893	Csi-2	0.984
Cde-3	0.733	Ctu-3	0.926	Csa-3	0.974	Csi-3	0.887
Cde-4	0.980	Ctu-4	0.896	Csa-4	0.924	Csi-4	0.929
Cde-5	0.693	Ctu-5	0.970	Csa-5	0.915	Csi-5	0.943
Cde-6	0.918	Ctu-6	0.877	Csa-6	0.958	Csi-6	0.856
Cde-7	0.843	Ctu-7	0.959				
Cde-8	0.868	Ctu-8	0.962				
Cde-9	0.792	Ctu-9	0.988				
Cde-10	0.903	Ctu-10	0.959				

Data analysis was performed by the professional software named Traditional Chinese Medicine Chromatographic Analyzing and Data Administration System (National Institute for the Control of Pharmaceutical and Biological Products) for evaluating the similarity of different chromatograms by calculating the correlation coefficient.

to each other and *C. sinensis* samples were somewhat different. It is well known that the standard HPLC fingerprint must be representative of authentic herb. For this purpose, the one with the biggest similarity was selected as the standard fingerprint. Accordingly, Cde-4 (*C. deserticola*), Ctu-1 (*C.*

Table 3. Sequences of 8 Polymorphic Primers Used for ISSR Fingerprint and the Amplified DNA Products

No.	Primer sequence	No. of amplified bands	No. of polymorphic bands	No.	Primer sequence	No. of amplified bands	No. of polymorphic bands
1	$(AG)_8CT$	11	11	5	$(AG)_8CC$	13	13
2	$(AG)_8CA$	15	15	6	$(GA)_8C$	10	10
3	$(GA)_8CT$	14	14	7	$(AG)_8TC$	11	11
4	$(GA)_8CC$	9	9	8	$(GA)_8T$	13	13

tubulosa), Csa-3 (*C. salsa*) and Csi-2 (*C. sinensis*) were selected as the standard fingerprints for different *Cistanche* species with 10, 7, 12 and 6 characteristic peaks, respectively. By comparison with standards, peaks with the retention time at 46.5 and 60.2 min were identified as echinacoside and acteoside. From Fig. 2, it is found that the fingerprint of *C. deserticola* was similar to *C. salsa*. Meanwhile, *C. tubulosa* can be differentiated from *C. deserticola* and *C. salsa* by comparing the relative contents of echinacoside and acteoside, which were dominant in *C. tubulosa*. Furthermore, echinacoside was the common peak in *C. tubulosa*, *C. deserticola* and *C. salsa*, which were not present in *C. sinensis*. Therefore, the presence of echinacoside can be distinguished *C. sinensis* from other *Cistanche* species.

As in ISSR fingerprints, ninety-four primers (synthesized by Sangon Inc., Shanghai, China) were tested for the identification of *Cistanche* plants, of which eight primers, namely primer 1—8 (Table 3), produced clear and reproducible bands. A total of 96 amplification bands were generated, which were all polymorphic ones. The loci numbers varied from 9 to 15 per primer (Table 3), with fragment size ranging from 200 to 2000 bp (Fig. 3). The eight ISSR fingerprints obtained using primers 1—8 were showed in Fig. 3. Primer 1 revealed a potential specie-specific band (Fig. 3A) for *C. sinensis* (lane 3; about 1800 bp). Primer 2 could be used to identify two *Cistanche* species (Fig. 3B), one specific band for *C. deserticola* (lane 1; about 2100 bp) and two for *C. tubulosa* (lane 4; about 1600 bp and 1300 bp). It is obvious to find a specific band for *C. salsa* (lane 2; about 2000 bp) by using primer 3 (Fig. 3C). Primer 4 could identify *C. deserticola* with a specific band (lane 1; about 1500 bp) (Fig. 3D). The specific bands included one for *C. deserticola* (lane 1; about 1100 bp), one for *C. tubulosa* (lane 4; about 510 bp), which were obtained using the primer 5 (Fig. 3E). Primer 6 could identify *C. deserticola* with a specific band (lane 1; about 1200 bp) (Fig. 3F). The specific bands included one for *C. salsa* (lane 2; about 450 bp), one for *C. tubulosa* (lane 4; about 220 bp), which were obtained using the primer 7 (Fig. 3G). Primer 8 could identify *C. sinensis* (lane 3; about 1600 bp) and *C. tubulosa* (lane 4; about 1400 bp) with specific bands (Fig. 3H). Complete distinctness of four species examined was achieved by the amplification pattern from primers 1—8, different species showing the presence of a band from those where the band was absent. To authenticate *Cistanche* species efficiently, an unweighted pair group method using arithmetic average (UPGMA) dendrogram (Fig. 4) was obtained by comparing the similarity among the four *Cistanche* species basing on primer 8. The dendrogram clearly distinguished the examined *Cistanche* species into four groups. In the first cluster, there are six *C. sinensis* samples with three sub-groups. The second cluster consisted of ten *C. tubulosa* samples with three sub-groups. Six *C. salsa* samples with two sub-groups formed the third cluster. The remaining ten *C. deserticola* samples formed the fourth cluster, which consisted of three sub-groups. From the UPGMA clustering results, *C. deserticola* and *C. salsa* showed the close affinity with a similar index value of 0.58. Meanwhile, *C. tubulosa* showed genetic relationship with *C. deserticola* and *C. salsa* at a similar index value of 0.44. In contrast, *C. sinensis* showed distant relationship with other three *Cistanche* species at a similar index value of 0.36. Interestingly, the cluster results by ISSR fingerprint is in accordance with the results generated by chemical fingerprint.

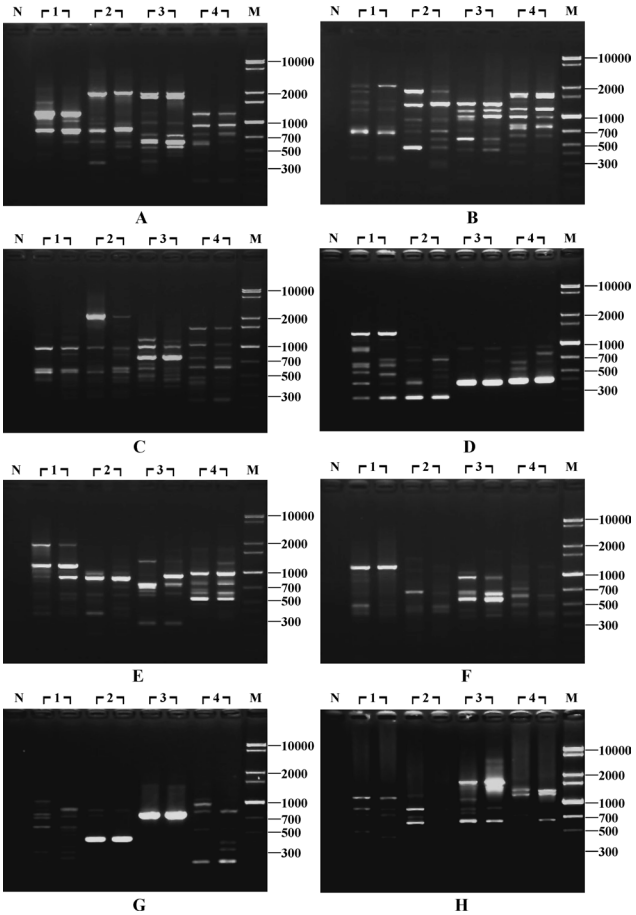

Fig. 3. ISSR Fingerprint Patterns Using the Primers 1—8 (A—H)

Lanes 1—4 represent *C. deserticola*, *C. salsa*, *C. sinensis*, *C. tubulosa*, respectively. Lane N: Blank. Lane M: 1 kb Plus DNA Ladder.

CONCLUSION

Precise identification of crude drugs is a prerequisite for chemical and pharmacological investigations of traditional

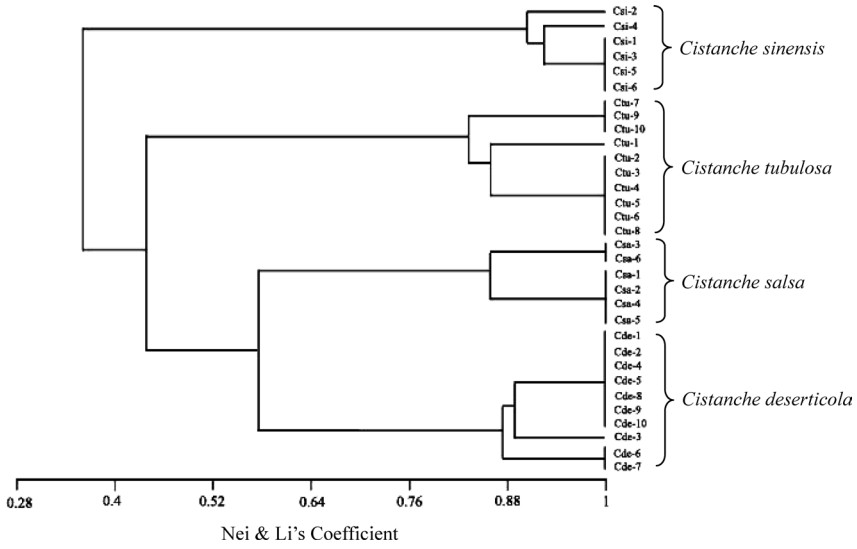

Fig. 4. UPGMA Drendrogram on the Basis of ISSR Fingerprints of Primer 8

Chinese medicine and for their clinical applications. HPLC fingerprint has played more and more important role for identification and quality control of TCM, which has been used as an effective tool for *Cimicifuga* species identification.[14] Molecular genetic methods have several advantages over classical authentication by using morphological and chemical analyses,[15] and effectively applied to discriminate *Astragalus* species.[16] In case of *Cistanche* species here, both chemical fingerprint and genetic analysis are used for identification, and the results show that chemical and genetic methods are two rather distinct analyses, but their results are in line with each other suggesting the unique characteristic of the species. Thus, both chemical and genetic methods could be used as tools in identifying the correct species of *Cistanche*. Meanwhile, our present study provides a powerful evidence for the rationality of *C. tubulosa* using as a new natural source of Herba Cistanches in Chinese Pharmacopoeia (2005 edition). In addition, our results suggest that *C. sinensis* should not be as substitute of *C. deserticola* and *C. tubulosa* due to the deficiency of echinacoside.

Acknowledgements We thank the National Natural Science Foundation of China (Grant No. 30371042) for financial support of this work. We are grateful to Dr. Christopher X. Lu for reading the manuscript.

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