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Full Length Research Paper

Molecular identification of Cistanches Herba and its adulterants based on nrITS2 sequence

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Cistanches Herba is a commonly used Chinese medicine for its tonification properties. However, it is often confused and substituted with the dried succulent stems of *Orobanche pycnostachya*, *Orobanche coerulescens*, *Boschniakia rossica*, *Cistanche salsa*, *Cistanche sinensis* and *Cynomorium songaricum*. Identifying Cistanches Herba based only on its morphological and chemical appearance is generally difficult. To develop a convenient and efficient identification method for discriminating Cistanches Herba and its adulterants, the second internal transcribed spacer (ITS2) of the ribosomal DNA of 39 individuals from Cistanches Herba and its adulterants were examined in this study. A phylogenetic tree was constructed using the neighbor-joining (NJ) and Kimura 2-parameter (K2P) methods. The results showed that the length of ITS2 sequence of *Cistanche deserticola* and *Cistanche tubulosa* were 229 and 233 bp, respectively. The intraspecific genetic distance of the two botanical origins of Cistanche Herba were both lower than their respective interspecific genetic distance with their adulterants. In the cluster dendrogram, *C. deserticola* and *C. tubulosa* were both monophyletic, and were distinguished from their adulterants. In conclusion, ITS2 can be used as a DNA barcode to efficiently identify Cistanches Herba and its adulterants, and can provide important information for quality evaluation, resource protection, exploitation, and sustainable utilization of this medicinal material.

Key words: Cistanches Herba, Orobanchaceae, internal transcribed spacer (ITS2), DNA barcode, molecular Identification.

INTRODUCTION

Cistanches Herba, which is obtained from the dried succulent stems of *Cistanche deserticola* Y. C. Ma and *Cistanche tubulosa* (Schrenk) Wight of Orobanchaceae, is widely used in traditional Chinese medicines to invigorate the kidney and strengthen yang sexuality, tonify the blood and essence, and loosen bowel to relieve constipation (Chinese Pharmacopoeia, 2010). It is known as the “ginseng of the desert”. However, because of the unique biological characteristics of its botanical origins as parasitic plants and the increasing demand for this medicine, its resources have become scarcer, often causing an imbalance between supply and demand. Cistanches herba is often misidentified and substituted with the dried succulent stems of *Cistanche salsa* (C. A. Meyer) Beck, *Cistanche sinensis* Beck, *Orobanche pycnostachya* Hance, *Orobanche coerulescens* Stephan, *Boschniakia rossica* (Cham. et Schlecht.) Fedtsch. et Flerov. of Orobanchaceae, and *Cynomorium songaricum* Rupr., which belonging to Cynomoriaceae (Cai and Li, 1999).

Previous studies on Cistanches Herba and its adulterants were mainly performed based on macroscopic and microscopic inspection and chromatographic profiling (Jiang et al., 2009; Liu et al., 2009). However, these methods have been more or less

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Abbreviations: IMPLAD, Institute of medicinal plant development; **ITS2,** the second internal transcribed spacer; **K2P,** Kimura 2-parameter; **MEGA,** molecular evolutionary genetics analysis; **NJ,** neighbor-joining.

affected by the users and the experimental condition. Thus, establishing a more reliable and accurate method for discriminating Cistanches Herba and its adulterants, and clarifying the confusion regarding this medicinal material are critical. DNA barcoding is a novel technique that uses a short, standardized DNA region to identify species (Hebert et al., 2003); it has become the trend and hot spot of biology systematics and identification (Schindel and Miller, 2005; Chen et al., 2009). This technique can consistently identify a species, regardless of the morphological features of the samples and the professional level of the users. Recently, the internal transcribed spacer (ITS2) sequence was proposed as a standard barcode for the authentication of medicinal plants (Chen et al., 2010; Gao et al., 2010a, b; Pang et al., 2010; Yao et al., 2010; Zhu et al., 2010). In the present study, we utilize ITS2 as a DNA barcode to discriminate Cistanches Herba and its adulterants, aiming to provide molecular evidence for its identification, exploitation, and sustainable utilization.

MATERIALS AND METHODS

Plant materials

The study materials comprised experimental samples and some related sequences from GenBank, which representing 39 samples and 8 species of Cistanches Herba and its adulterants (Table 1). The experimental materials were identified by Professor Jun Chen and Professor Yulin Lin from the Institute of Medicinal Plant Development (IMPLAD). The voucher samples were deposited in the herbarium of IMPLAD.

DNA extraction

Genomic DNA was extracted from the silica gel-dried succulent stems or the medicinal materials bought from the markets. About 10 mg of each dried tissue was rubbed for one minute at a frequency of 30 times/s in a FastPrep bead mill (Retsch MM400, Germany). DNA extractions were performed using a Plant Genomic DNA Kit (Tiangen Biotech Co.), following the manufacturer's instructions. PCR amplification and sequencing

The ITS2 region was amplified by polymerase chain reaction (PCR) with primers as follows: forward primer, 5′-GCGATACTTGGTGTGAAT-3′; and reverse primer, 5′-GACGCTTCTCCAGACTACAAT-3′. A 25μL PCR amplification was performed according to the procedure provided by Chen et al (Chen et al., 2010). The purified PCR products were sequenced in both directions with the primers used for PCR amplification on a 3730XL sequencer (Applied Biosystems).

Sequence alignment and data analysis

Contig assembly and generation of consensus sequences were performed using CodonCode Aligner V 3.0 (CodonCode Co., USA). The bilater primers were removed. The ITS2 sequences were annotated and trimmed using ITS2 annotation tools based on the Hidden Markov Model (Keller et al., 2009). Two conserved regions of the 5.8 and 28 S gene were used to delimit the ITS2 region. All sequences were then aligned using Clustal W (Thompson et al., 1994) and the genetic distances were computed using MEGA 4.0 software according to the K2P model (Tamura et al., 2007). The phylogenetic tree was built using NJ method. A bootstrap test with 1000 replicates was applied to assess the reliability of the phylogenetic trees.

RESULTS

Sequences comparison

A total of 39 samples representing 8 species of Cistanches Herba and its adulterants were investigated in the current study. Of these, 28 ITS2 sequences were obtained after our experiment, whereas 11 ITS2 sequences were downloaded from GenBank. The length of the ITS2 sequences of *C. deserticola* was 229 bp, including one Poly G; and the GC contents were 55.0%. After alignment, the sequences of 12 samples of *C. deserticola* from different localities were identical, so the intraspecific genetic distance of *C. deserticola* was 0.000. The length of the ITS2 sequences of *C. tubulosa* ranged from 229 to 233 bp, including one Poly C and one Poly G; and the GC contents were 54.7 to 59.0%. After alignment, the sequences of 8 samples of *C. tubulosa* from our experiment were identical, but different from the 2 samples downloaded from GenBank with 15 sites variation. Among them, 11 sites were base transitions and 4 were base transversions. The intraspecific genetic distance was calculated according to the K2P model. The maximum intraspecific variation was 0.074, whereas the average was 0.026.

The ITS2 sequences of *C. deserticola* and its adulterants were 244 bp long after alignment, and the average of its interspecific divergence was 0.505, while the minimum was 0.009. The ITS2 sequences of *C. tubulosa* and its adulterants were also 244 bp long after alignment. The average of the interspecies divergence was 0.534, whereas the minimum was 0.238. Among them, *C. salsa* has the closest genetic distance with *C. deserticola*, which was only 0.009.

Phylogenetic analysis

To evaluate the ability of the ITS2 sequence in identifing Cistanches Herba, a phylogenetic tree was constructed using the NJ method based on MEGA 4.0 software. The phylogenetic tree presents 39 ITS2 sequences representing 8 species of Cistanches Herba and its adulterants (Figure 1). Different samples from the same species can be seen in the same clade. Both *C. deserticola* and *C. tubulosa* are easily distinguishable from their substitutes and spurious breeds. In the cluster dendrogram, the species of *Cistanche* examined were clustered into three groups. The first was *C. sinensis*, the second comprised *C. deserticola* and *C. salsa*, and the third was *C. tubulosa*. In addition, *C. songaricum* was separated from other species first, so it was the farthest among the examined species.

Table 1. Source of materials.

Material	Voucher	Collection place	GenBank No.
Cistanche deserticola	PS1687MT01	Yongning, Ningxia	GQ434850
C. deserticola	PS1687MT02	Alxa right banner, Inner Mongolia Autonomous Region	JF915377
C. deserticola	PS1687MT03	Alxa left banner, Inner Mongolia Autonomous Region	JF915378
C. deserticola	PS1687MT04	Alashan market, Inner Mongolia Autonomous Region	JF915375
C. deserticola	PS1687MT05	Alxa left banner, Inner Mongolia Autonomous Region	JF915376
C. deserticola	PS1687MT06	Alxa left banner, Inner Mongolia Autonomous Region	JF915372
C. deserticola	PS1687MT07	Alxa left banner, Inner Mongolia Autonomous Region	JF915370
C. deserticola	PS1687MT08	Alxa left banner, Inner Mongolia Autonomous Region	JF915373
C. deserticola	PS1687MT09	Qingping market, Guangdong Province	JF915374
C. deserticola	PS1687MT10	Qingping market, Guangdong Province	JF915387
C. deserticola	PS1687MT11	Dengkou, Inner Mongolia Autonomous Region	JF915371
C. deserticola	-	-	AB217873
C. tubulosa	PS0811MT01	Yutian, Xinjiang Autonomous Region	GQ434562
C. tubulosa	PS0811MT02	Hangzhou, Zhejiang Province	GQ434563
C. tubulosa	PS0811MT03	Hohhot, Inner Mongolia Autonomous Region	JF915383
C. tubulosa	PS0811MT04	Dengkou, Inner Mongolia Autonomous Region	JF915384
C. tubulosa	PS0811MT05	Dengkou, Inner Mongolia Autonomous Region	JF915385
C. tubulosa	PS0811MT06	Dengkou, Inner Mongolia Autonomous Region	JF915386
C. tubulosa	PS0811MT07	Dengkou, Inner Mongolia Autonomous Region	JF915379
C. tubulosa	-	-	AB217871
C. tubulosa	-	-	AY209298
C. tubulosa	-	-	AY209299
C. salsa	PS2002MT01	Yanchi, Ningxia Autonomous Region	JF915380
C. salsa	-	-	AB217872
C. sinensis	PS2005MT01	Yanchi, Ningxia Autonomous Region	JF915388
C. sinensis	PS2005MT03	Fengshaqu, Ningxia	JF915390
C. sinensis	PS2005MT04		JF915389
C. sinensis	-	-	AB217874
Boschniakia rossica	PS2004MT01	Fusong, Jilin Province	JF915392
B. rossica	PS2004MT02	Fusong, Jilin Province	JF915381
B. rossica	-	-	AY911214
Orobanche coerulescens	PS2006MT01	Yanchi, Ningxia Autonomous Region	JF915391
O. coerulescens	-	-	AY881142
O. coerulescens	-	-	AY209235
O. pycnostachya	PS2006MT02	Donglin mountain, Beijing	JF915382
O. pycnostachya	-	-	AY881143
Cynomorium songaricum	PS1688MT01	Alashan market, Inner Mongolia Autonomous Region	JF915369
C. songaricum	PS1688MT03	Yanchi, Ningxia Autonomous Region	JF915368
C. songaricum	-	-	HQ222981

DISCUSSION

Cistanches Herba is a famous tonifying agent in Chinese medicine, ranked “high grade” in the Shennong Bencao Jing. With the resource shortage, many substitutes and adulterants have emerged in different regions of China, thus causing confusion in the market and influencing its quality and clinical medication. In this study, ITS2 sequence was utilized as a DNA barcode to discriminate Cistanches Herba and its adulterants for the first time.

We found that the ITS2 sequences of *C. deserticola* and *C. tubulosa* were 229 and 233 bp, respectively, and were easily amplified. The intraspecific genetic distance of the two species were both lower than their interspecific genetic distance with their adulterants. In the cluster dendrogram, *C. deserticola* and *C. tubulosa* were both monophyletic, which indicated that they were distinguishable from their adulterants. Thus, using the ITS2 region as a DNA barcode is suitable for the identification of Cistanches Herba and its adulterants.

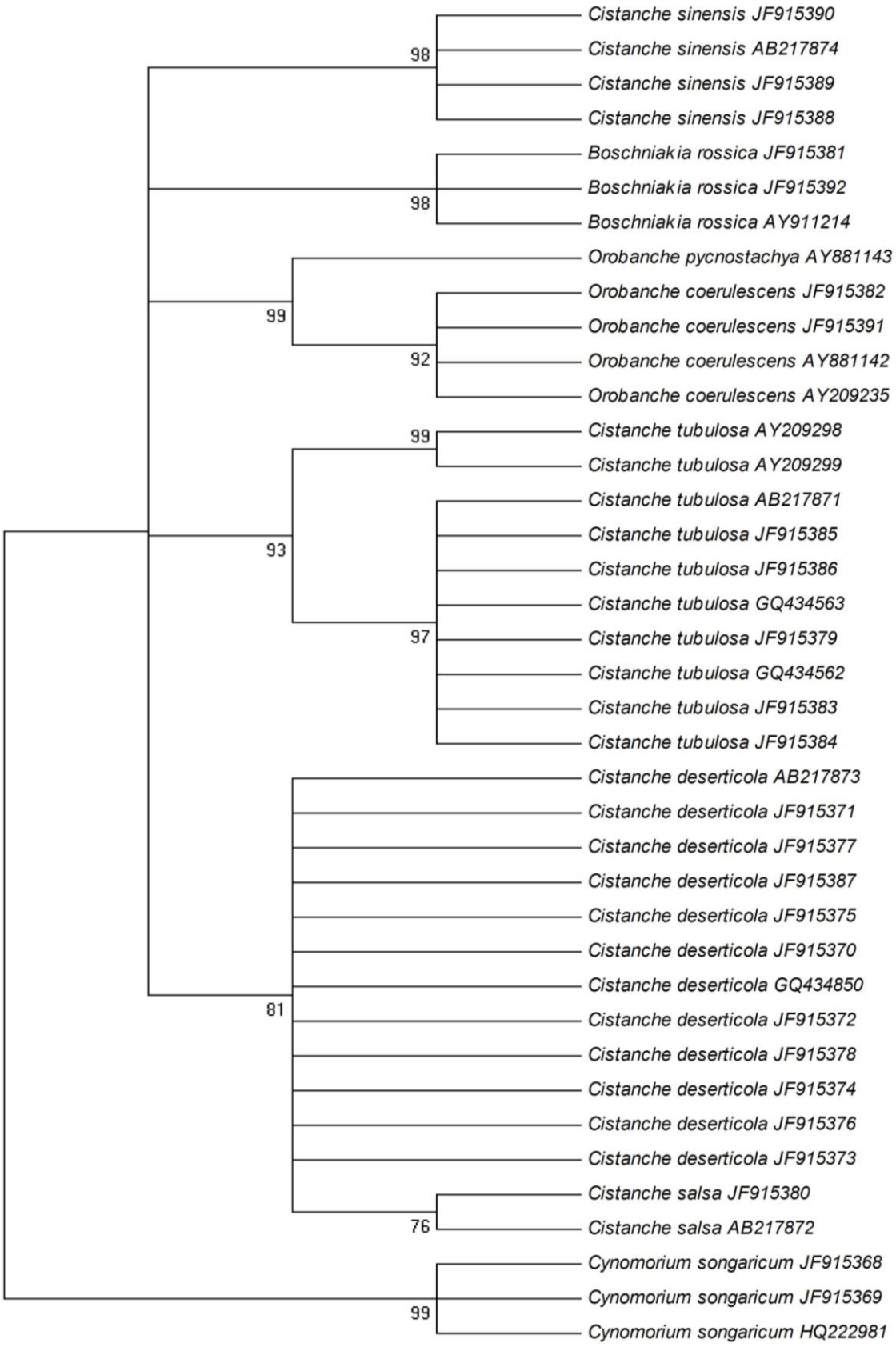

Figure 1. Phylogenetic tree of Cistanches Herba and its adulterants constructed from the ITS2 sequences using NJ method. The bootstrap scores (1000 replicates) are shown (≥60%) for each branch.

Han et al. (2010) employed the chloroplast *psbA-trnH* intergenic spacer region to identify *Cistanches*. Their results indicated that *psb*A-*trn*H represented a barcode that could be used to identify *Cistanche* species and other morphologically undistinguishable species. In contrast to the length of the *psb*A-*trn*H sequence (about 580 bp), the length of the nuclear ITS2 sequence was only about 230 bp in our study. Thus, the latter was relatively shorter and more easily amplified, especially to degraded samples. Furthermore, in heredity, the nuclear gene comes from the parents, but the chloroplast genome only comes from maternal inheritance. Therefore, the nuclear sequence has predominance in reflecting the true relationship among the different species (Zhang et al., 2009).

The botanical origins of Cistanches Herba were *C. deserticola* and *C. salsa* recorded in the compendiums of materia medica of the past dynasties in China. In addition, the assaying of echinacoside and actaea spicata glycoside was performed using high-performance liquid chromatography, and the result showed that *C. salsa* had the highest content of the two active ingredients among the investigated samples of Cistanches Herba. By comparison of traditional pharmacodynamic action of medicinal materials of *C. deserticola*, *C. tubulosa* and *C. salsa*, *C. salsa* had the best effect in invigorating the kidney (Cai and Li, 1999). Therefore, these studies provided scientific evidence for the usage of *C. salsa* as medicine. Based on our result, *C. deserticola* and *C. salsa* had the closest relationship, thus providing molecular evidence for *C. salsa* as medicine and it can be served as the reasonable substitutes of Cistanches Herba. In recent years, the wild resources of Cistanches Herba have been nearly exhausted, *C. deserticola* and its host-*Haloxylon ammodendron* (C. A. Mey.) Bunge have been listed as second order preserved plants in China. *C. salsa* is mainly distributed near deserta, lake basins, lowlands, and high salty-alkali places in Inner Mongolia, Ningxia, Gansu and Xinjiang. Its common hosts include *Kalidium foliatum* (Pall.) Moq., *Reaumuria songarica* (Pall.) Maxim., *Salsola passerina* Bunge, *Nitraria tangutorum* Bobr. and *Achnatherum splendens* (Trin.) Nevski. It is well known that there are vast expanses of saline lands in China. If adaptation to local conditions to grow the host plants of *C. salsa*, we will not only improve the quality of the saline soil, but also harvest the medicinal materials.In conclusion, ITS2 is preferable as a DNA barcode for identifying Cistanches Herba and its adulterants, and it can provide important theoretical basis for the reasonable exploitation and sustainable utilization of the resources of Cistanches Herba.

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