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Molecular, Chemical and Microscopic Analysis of Medicinal Plant (*Cymbopogon distans*) from Its Adulterants

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Abstract: Morphological resemblance among *Cymbopogon distans* species and their adulterants which are procured from different markets in the form of dried or fresh plant tissues represents a serious problem for quality and safety of medicinal plants, as it supports frauds for substitution. In order to assure the quality control of *C. distans* species, DNA barcode, microscopic identification and High Performance liquid chromatography (HPLC) fingerprint were synergistically used to discriminate *C. distans* from its adulterants. In this work, the internal transcribed spacer 2 (ITS2) was chosen for distinguishing *C. distans* from their usual adulterants from 5 provinces of China. Sequences were obtained after removal of the 5.8S and 28S sections. A multiple sequence alignment was finalized. Results exhibited that ITS2 performed well, with 100% of genera being accurately distinguished. Additionally, finding indicates that the upper epidermis in leaf of *C. citratus* was composed of one layer of wide elongated cells called Bulliform cells whereas in *C. distans* the upper epidermis consist of one layer cell, thus these feactures are very important for the anatomy identification. The HPLC fingerprint method was also developed, the similarities of 6 batches of *C. distans* samples were all more than 0.93, indicating that the samples from different geographical origins shared similar HPLC fingerprints. And the similarities between *C. distans*, *C. citratus, C. flexuosus* and *Imperata cylindrica* were all less than 0.93, suggesting that there was significance difference between *C. distans* and its adulterants. Finally, it was concluded that the DNA barcode, HPLC fingerprint and microscopic methods could effectively authenticate the quality of *C. distans* from their adulterants and can provide accurate and reliable information to tackle the complex quality issue of *C. distans* in markets. This is the first report of detailed analysis of the *C. distans* for effective quality and safety.

Keywords: Internal Transcribed Spacer (ITS2), DNA barcode, *Cymbopogon distans*, High Performance Liquid Chromatography (HPLC)

1. Introduction

Cymbopogon, commonly known as lemongrass in English, is one of the most important medicinal plants of the family Poaceae [1]. The genus *Cymbopogon* encompasses 140 species mainly distributed in tropical and semi-temperate regions of Asia, South America, Autstralia and Africa. It is extensively recognized for producing high value aromatic oils and important components such as allantoin for industries, which find large use in perfumery, cosmetics, and in the pharmaceutical industries [1-3].

Cymbopogon species exhibits a large variation in morphological characteristics and essential oil composition at inter and intra specific levels [4]. In general, *Cymbopogon distans* medicinal materials are collected from wild, making its quality of unstable. Traditionally, *C. distans* and its adulterants were discriminated mainly using morphological characteristics by experienced or trained experts. However, it is somewhat difficult to distinguish them merely based on morphological characteristics [5-8]. It is therefore important to emphasize that the identification of *C. distans* are increasingly being seen as a major challenge, which poses quality problem to both pharmaceutical industries and consumers. Hence, establishing a

more reliable and accurate method for discriminating *C. distans* from its adulterants, and illuminating the confusion regarding this medicinal material become urgent and acute. Bearing in mind the adverse effects of adulterants, attention has been shifted to the potential use of DNA barcoding to investigate the extent of adulteration in products.

DNA barcoding is a new molecular diagnostic technique for species identification that has potential to overcome the above mentioned difficulties. Compared with the traditional methods, the DNA barcoding technique gives consistent and reliable outcomes in spite of the age, plant part or environmental factors of the sample [9]. It makes the results more rapid, subjective and accurate. Since its discovery, the power of DNA barcoding has opened up new areas in taxonomic, biological, ecological, and evolutionary research by assisting species identification. In plants, the commonly used primers, namely Internal transcribed spacer (ITS), ITS2, maturase K (matK) gene, ribulose 1,5, bisphosphate carboxylase (rbcL) gene, trnL-F, rpoC1, and (trnH–psbA) Intergenic Spacer have been widely used as potential plant identification and authentication markers for medicinal plant materials, either individually or in combination [10-13]. Recently, Chen and his colleagues [14] have contributed to the DNA barcode database through their recent outstanding research work on the identification of herbal materials based on the ITS2 and psbA-trnH barcodes, and consequently, ITS2 has been validated as a DNA barcode for medicinal plants and psbA-trnH region proposed as a complementary barcode and are now recommended as the universal DNA barcode for plants [15]. At the same time, the China Plant BOL Group [16] analyzed the efficacy of ITS2 as a plant DNA barcode across a huge sample size and also officially recommended that ITS2 regions can serve as a standard plant barcode for distinguishing species. Additionally, the use of high performance liquid chromatography (HPLC) fingerprint emerges to be the popular method utilized in the quality issue of species because of its convenience, large suitability, stability of the sample compound, high accuracy and reproducibility and has been accepted by many organizations U.S. Food [17] and Drug administration [18], and European Medicines Agency [19]for the assessment of the quality of botanical products and as the common method in Chinese pharmacopoeia.

Up to now, most studies of *C. distans* have been focused on the chemical composition of the essential oil of *C. distans* [20-22]. No previous reports have, to the best of authors' knowledge, published regarding the molecular, chemical fingerprint and microscopic analysis of *C. distans* from its adulterants for quality and safety of medicinal plants. In this context, these three techniques were evaluated to identify *C. distans* and its adulterants. The first focus concerns the anatomical and morphology attributes of the leaves of *C. distans*, and differentiates the differences among species. Second, the suitable and feasible DNA barcode were assessed to accurately discriminate between *C. distans* and its adulterants to ensure their safe application in medical use and rational development and utilization, thus providing theoretical basis and data support. Finally, a comprehensive and quantifiable identification HPLC method were developed for the assessment of different batches of *C. distans* species. Additionally, the similarity evaluation between chromatographic fingerprints was carrying out to assure the quality control of *C. distans.*

2. Materials and Methods

2.1. Identification of C. distans by Morphological and Anatomical Analysis

The fresh plant materials namely *C. citratus*, *C. distans* and *C. flexuosus* collected from different provinces of China, were identified using the measurement given in flora of China by Chen et al. [25] for morphological and anatomical identification. Briefly, the leaves were segmented and fixed for 24 hours using the standard fixative formaldehyde, acetic acid and ethanol (FAA), embedded in paraffin wax then sectioned [23-24] and stained by flooding them into Ethanol plus 0.8g fast green and finally dehydrated back. The slides were analyzed using Light microscope Olympus DP72 China; the objective lenses were (x4, x10 and x25). Microtome QPJ-1B China were used for the segments sectioning, Oven Shanghai China. The reagents used were from Tianjin Zhi Yuan Reagent Co. Ltd. China.

2.2. Molecular Identification of C. distans

2.2.1. Material Collection

A total of 32 samples including 6 specimens of *C. distans* were gathered from different geographical areas in China, as reported in (Table 1). 15 samples from the three adulterants species of *C. citratus*, *C. flexuosus* and *Imperata Cylindrica* were collected from Yunnan, Anhui, Hebei, Sichuan, Guanxi, and Guangdong Provinces, China (Table 1). The remaining 11 sequences were downloaded from Genbank and the obtained specimens were identified by Song Liangke Associate Professor at the School of Life Science and Engineering, Institute of Southwest Jiaotong University

Table 1. *Details of the market samples of C. distans collected in different provinces of China and its adulterants.*

Name of the species	No.of Sample	Origins	Genbank Accession no. of ITS2
Cymbopogon distans	6	Yunnan, Sichuan	OUXK11163 http://www.iflora.cn. China
Cymbopogon goeringii	3	Genbank	KF163608.1,KF163609.1,KF163607.1
Cymbopogon citratus	9	Genbank, Hebei, Guangdong, Yunnan, Anhui, Sichuan	AF019823.1
Cymbopogon flexuosus	11	Sichuan, Hebei, Anhui, Guangxi, Genbank	KX828250.1, KX828246.1,KX828244.1, KX828243.1,KX828245.1
Cymbopogon martini	1	Genbank	DQ005037.1,
Imperata cylindrica	2	Genbank, Sichuan	KF163640.1,

2.2.2. DNA extraction, PCR Amplification and Sequencing

DNA from dried leave samples (30 mg) was extracted as described by the protocol of Plant Genomic DNA Kit (Tiangen Biotech, Co., Ltd., Beijing, China). The concentration and the purity of DNA were determined electrophoretically by 1.0 % agarose gels. The DNA sample was then diluted to the concentration of 20 ng/µl and stored at -20°C. The primers used were as follow: Forward primer: 5'-ATGCGATACTTGGTGTGAAT-3'; and Reverse primer: 5'-GACGCTTCTCCAGACTACAAT-3', the PCR amplification conditions were set as follow: initially at 94°C for 5 min; followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 45 s with a final extension at 72°C for 10 min. The PCR amplification mixtures for ITS2 region was as follows: 25µl of 2 xTag PCR Master Mix, 8µl of genomic DNA, 1µl of each primer and 15µl of ddH_2O in a total volume of 50 µl (Table 2).PCR amplification of the marker was performed with a thermal cycler (Applied Biosystems). The PCR products were assessed electrophoretically by 1.0% agarose gels.

Sequences were edited and assembled using clustal W (codon) and refined manually. ITS2 regions were identified and delimited using a website for Hidden Markov Model (HMM) based ITS2 delineation (http://its2.bioapps.biozentrum.uni-wuerzburg.de/). Genetic distances were calculated using the Kimura-2-Parameter (K2P). All the newly obtained ITS2 sequences were uploaded to GenBank. ITS2 sequences were subjected to BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) for identification of the sequences at species level. The Phylogenetic tree was constructed with the Neighbor-Joining method by using MEGA 5.0; the bootstrapping was done with 1000 replications.

2.3. HPLC Analysis of C. distans

2.3.1. Plant Materials, Sample and Standard Solution Preparation

The plant materials were collected from different locations as mentioned above (Table 1). As for sample preparation, briefly, each of the collected samples of *C. distans, C. flexuosus, C. citratus and Imperata cylindrica* was oven-dried until constant weight was reached, and then powdered, sieved through the 40-mesh. 2.0 g of the powdered samples was precisely weighted and then put into a volumetric flask containing approximately 100 ml of methanol solution for 48h, the solution was then placed in ultrasonic cleaner for 30 min and was filtrated through an analytical filter paper and the slurry mixture was centrifuged (10,000 rpm/min) for 10 min. The supernatant was collected and methanol was added to make the weight lost and finally the sample was filtrated through 0.45µm syringe filter prior to HLPC analysis.

Stock solution was prepared by dissolving accurately weighted standards in absolute methanol. Standard solutions were prepared by serial dilution of the stock solutions to the mobile phase working range of each substance. All of the standard solutions were then stored at 4°C and brought to room temperature prior to use. The standard solutions were then filtered through 0.45µm membrane prior to HPLC analysis. The batches number of all the plant materials are presented as follows: 7 species of *C. citratus* (S1-S2) and 6 species of *C. distans* (S15, S16, S17 and S20) were originated from Yunnan province; S3, S9, S13, one species of *Imperata cylindrica* (S14), and S18 and S19 from Sichuan province; (S4-S5) from Guangzhou Province; S6 and (S10-S11) from Hebei province; S7 and (S12) from Anhui province.

2.3.2. HPLC Instrumentation and Chromatographic Conditions

All HPLC analyses were carried out on an Agilent 1260 Series HPLC-DAD system consisting of a vacuum degasser, pumps, auto sampler, column compartment and DAD detector. The conditions of solvent gradient elution was set as follows: 0-10 min, 10% B-90% A; 10-15 min, 15% B-85% A; 15-35 min, 30% B-70% A; 35-55 min, 55% B-45% A; 55-60 min, 80% B-20% A; 60-70 min, 100% B-0% A. During the experiment, the chromatogram was monitored at a wavelength of 224 nm and the column temperature was kept at 30°C, while the injection volume of each sample and standard solution was 10µL. The HPLC mobile phase consisted of B acetonitrile – A 0.1% phosphoric acid, pH: 3 at a flow rate of 0.8 mL/ min; was prepared fresh daily, filtered then degassed prior to injecting.

2.3.3. Validation Procedure

The calibration curves were calculated by plotting the peak area (y) versus nominal concentration of each analyte (x) and were fitted to a linear function of type $y = ax + b$. The limit of detection (LOD) was evaluated as the minimum concentration of the compounds required to produce signals. Several analytical parameters were assessed to validate the HPLC method, i.e., based on the recommendations of ICH (International Council for Harmonization) guidelines for method validation. The accuracy tests were carried out by spiking the known contents of mixed standard solution into the known concentration of *C. distans* samples, and the evaluation was completed by analyzing the three different spiking concentrations of analytes in triplicates. The percent recovery rates for the analytes were presented as mean (100%).

2.3.4. Data Analysis

All determinations were conducted in triplicates with data reported as mean± standard deviation. While the data analysis was carried out using the SOP of Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A) which was developed by Chinese Pharmacopoeia Committee. This software assesses similarity based on correlative coefficient calculations for fingerprint chromatograms. Hierarchical cluster analysis (HCA) was carried out using SPSS software version 16, USA. The peaks areas of all the samples of *C. distans* were systematically clustered and the Ward method and Squared of the Euclidean distance were selected as a measure of similarity between samples of different batches. Reference chromatogram R was regenerated

with the aid of fingerprint chromatogram similarity calculation software.

3. Results

3.1. Microscopic Identification

The morphological characteristics were done by examining the species with naked eye and the leaf blade of all the samples was measured using a ruler and the mean was evaluated based on the width and length of the leaf blade. In the current study the morphological and anatomical features were used based on the description given in Flora of China (http://frps.eflora.cn/) [25].

The results demonstrate that the color of fresh species of *C. distans* was dark green color on upper and lower surface and the color of dried species *C. distans* was green pale with leaf blades narrowly linear, folded with a softer texture. The length and width of 6 species of *C. distans* were in the range of 35 to 50 cm, and from 1.7 to 4 mm, respectively (Figure 1C). *C. citratus* has simple leaf, alternate and linear. Their length and width were in the range of 62 to 70 cm and from 2 to 5 mm, respectively while having sheathed and apex acute (Figure 1A); it has a color of light green on upper and lower surface and has an entire margin and parallel venation compared with the morphology of *C. flexuosus* as the leaves length range from 82 to 120 cm, and the width from 5-19 mm (Figure 1B). The leaves size of *C. distans* was smaller than *C. citratus* and *C. flexuosus*, the venation of *C. citratus* and *C. flexuous* was parallel but the venation of *C. distans* was folded while the length and the width of *Imperata cylindrica* range from 50 to 100 cm and from 0.8-2 cm, respectively (Figure 1D).

In addition, the anatomy characteristic of *C. distans* show that upper epidermis in *C. distans* is built of one layer of miniature cells whereas upper epidermis in *C. citratus* gives one layer of wide cells. Both species possess mesophyll tissue and the spongy parenchyma is constituted of 1-2 layers in *C. citratus* followed by the upper epidermis, while the lower epidermis in *C. distans* is constituted of one layer of small cell following by the spongy mesophyll that is situated between vascular bunbles (Figure 2C). The vascular bundles in *C. citratus* are surrounded by 1-2 layers of sclerencyma cells but in *C. distans* the vascular bundles are covering by 2-3 layers of sclerencyma cells. The lower epidermis is formed of a layer of small cells in *C. citratus* followed by the vascular bundles (Figure 2A, B). This study demonstrated that the morphology and anatomy characteristics of the species of *C. distans* could be primarily discriminated from its adulterants (Table 2)

Figure 1. *Morphologies traits of C. citratus (A), C. flexuosus (B), C. distans (C) andImperata cylindrica (D) (Photograph taken by Kavidia Muyembe Diana).*

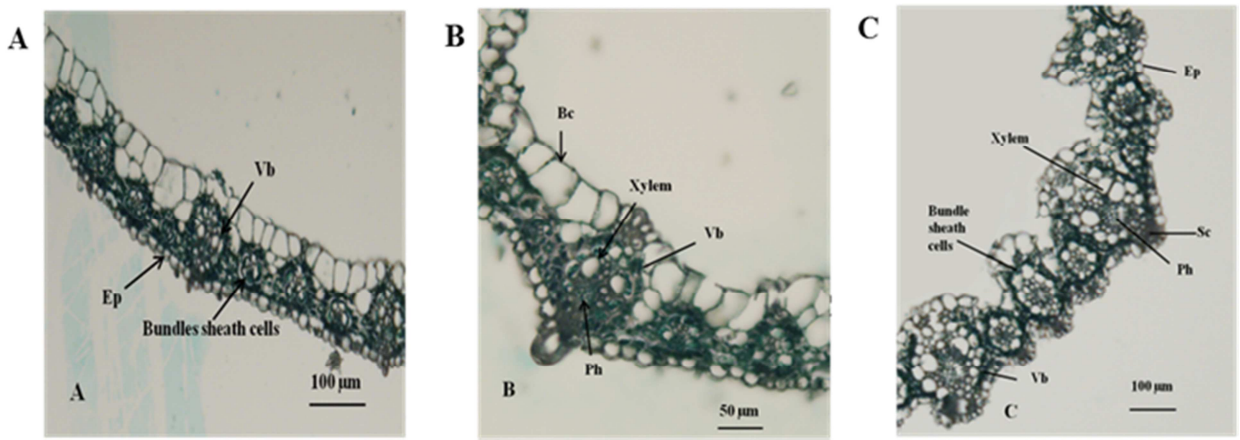


Figure 2. *The cross section of the leaf of C. distans (C) and leaf section of C. citratus(A, B) shows, xylem(x) and phloem (p), epidermis (ep), bundle sheath cells and vascular bundles (vb) bulliform cells (bc).*

Table 2. *Comparatively morphological characteristics of C. distans and its adulterants.*

	***C. citratus* (A)**		***C. distans* (C)**	
Characteristics	**Sample**	**Flora of China**	**Sample**	**Flora of China**
Leaf shape	Linear	linear-lanceolate	Narrow linear	Narrow linear
Leaf size/Length/width	62-70 cm /2-5 mm	30- 90 cm /5-15 mm	35-50 cm/1.7-4 mm	10-30(-50) cm/ 1.5-5 mm
Venation	Parallel	Parallel	Folded	flattened or folded
Color	Light green	pale green	Dark green	_

Table 2. *Continued.*

	***C. flexuosus* (B)**		***Imperata cylindrica* (D)**	
Characteristics	**Sample**	**Flora of China**	**Sample**	**Flora of China**
Leaf shape	Linear	Linear	Narrow linear	Narrow linear
Leaf size/Length/width	82-120 cm/5-1.9 cm	Up to100 cm/1.5 cm	50-100 cm/0.8-2 cm	20-100cm/0.8 -2cm
Venation	Parallel	Parallel	Parallel	Parallel
Color	Pale green	_	Pale green	_

3.2. DNA Barcode Analysis

3.2.1. Sequence Analysis

In the current study, the efficiency of the PCR amplification of ITS2 region was 100%. Moreover, the candidate barcode region was successfully sequenced by 100%. All of the sequencing outcomes were submitted to the Genbank database. The variations in the ITS2 sequences among *C. distans* and its potential adulterants were assessed. The ITS2 sequences length of *C. distans* was 219 bp. Furthermore, the other three common adulterants, *C. citratus*, *C. flexuosus* and *Imperata cylindrica* were between 219 to 224 bp in length. The guanine-cytosine (GC) content of the *C. distans* was 71.23% while the other three adulterants displayed GC contents which ranged from 71.4 to 73.1 % (Table 3).

Identification of polymorphic sites of ITS2 sequences exposed a significant sequence variation between *C. distans* and its adulterants species. One polymorphic site was identified between *C. distans* and *C. flexuosus* (position 48) and 5 indels (positions 23, 37-40) were identified in the alignment between these two species, five polymorphic sites were found between *C. distans* and *C. citratus* (positions 33,51, 134, 170, 194) (Table 4), while 9 polymorphic sites were located between *C. distan* and *imperata cylindrica* at the positions (33, 55, 62, 104, 154, 163, 175, 177 and 204), two polymorphic sites were identified between *C. distans* and *C. georigii* (positions 48, 196) and 4 indels (positions 37-40), There were no mutation among *C. distans* and *C. flexuosus*, whereas two mutations A-C and A-C were identified in *C. citratus* ITS2 sequences at the position 208 and 160, respectively.

Table 3. *ITS2 Sequences length and CG content % of C. distans sample and its closely related species.*

Sample No.	**Species**		**Sequence length bp**	
		Sample size	**ITS2**	**CG content %**
1	*C. citratus*	9	219	72.15
2	*C. distans*	6	219	71.23
3	*C. flexuosus*	11	223_224	71.15_71.43
4	*C. georigii*	3	223_224	70.85_70.09
5	*C. martini*	1	220	71.36
6	*Imperata cylindrica*	2	219	73.06

Table 4. *Single nucleotide polymorphisms of ITS2 DNA sequences among C. distans and its adulterants.*

Species	**23**	**33**	**37**	**38**	**39**	**40**	**48**	**51**	**55**	**62**	**104**	**134**	**154**	**163**	**170**	**175**	**177**	**194**	**196**	**204**
C. distans	_	T	_	_	_	_	T	T	C	T	T	A	T	A	C	C	A	T	G	A
C. flexuosus	C	T	T	A	G	G	C	T	C	T	T	A	T	A	C	C	A	T	G	A
C. citratus	_	G	_	_	_	_	T	C	C	T	T	G	T	A	T	C	A	A	G	A
Imperata cylindrica	_	G	_	_	_	_	T	T	A	C	C	A	C	G	C	T	T	T	G	C
C. georigii	_	T	T	A	G	G	C	T	C	T	T	A	T	A	C	C	A	T	A	A
C. martinii	A	G	_	_	_	_	T	C	C	T	T	A	T	A	C	C	T	T	G	A

3.2.2. The K2P Genetic Distance

The genetic distance matrix based on the ITS sequences was computed according to the Kimuna 2- parameter model (K2P) (Table 5). The K2P genetic distances among *C. citratus* species was 0.000 to 0.009 with an average distance of 0.002, meanwhile the genetic distance among *C. distans* was zero. The distance between *C. distans* and *C. flexuosus* ranged from 0 to 0.005, with an average distance of 0.002. And the distance between *C. distans* and *C. citratus* ranged from 0.000 to 0.033 with an average distance of 0.013. The inter-specific distance between *C. distans* and its adulterant ranged from 0.002 to 0.016 with an average distance of 0.006. Results also

displayed that the minimum inter-specific K2P distances between *C. distans* and its adulterant were larger than the maximum intra-specific distance among *C. distans* (Table 5). According to the Neighbor-Joining (NJ) tree (Figure 3), *C. distans* and its adulterants could be placed in genus *Cymbopogon* and differentiated from most of the other genera, whereas *C. distans* and its adulterants could be distinguished from each other based on the sequence divergences. Phylogenetic analysis using ITS2 sequences clearly distinguished the *C. distans* and the closely related species.

Table 5. *Intra-specific and inter-specific genetic distances between C. distans and its adulterants.*

Specie5	K2Pdistance	Average
C. distans	0.000-0.000	0
C. flexuosus	0.000-0.000	0
C. citratus	0.000-0.009	0.002
C. distans_C. flexuosus	0.000-0.005	0.002
C. distans_C. citratus	0.000-0.033	0.013
C. distans and its adulterants	0.002-0.016	0.006
C.distans and closely related species	0.005-0.034	0.011

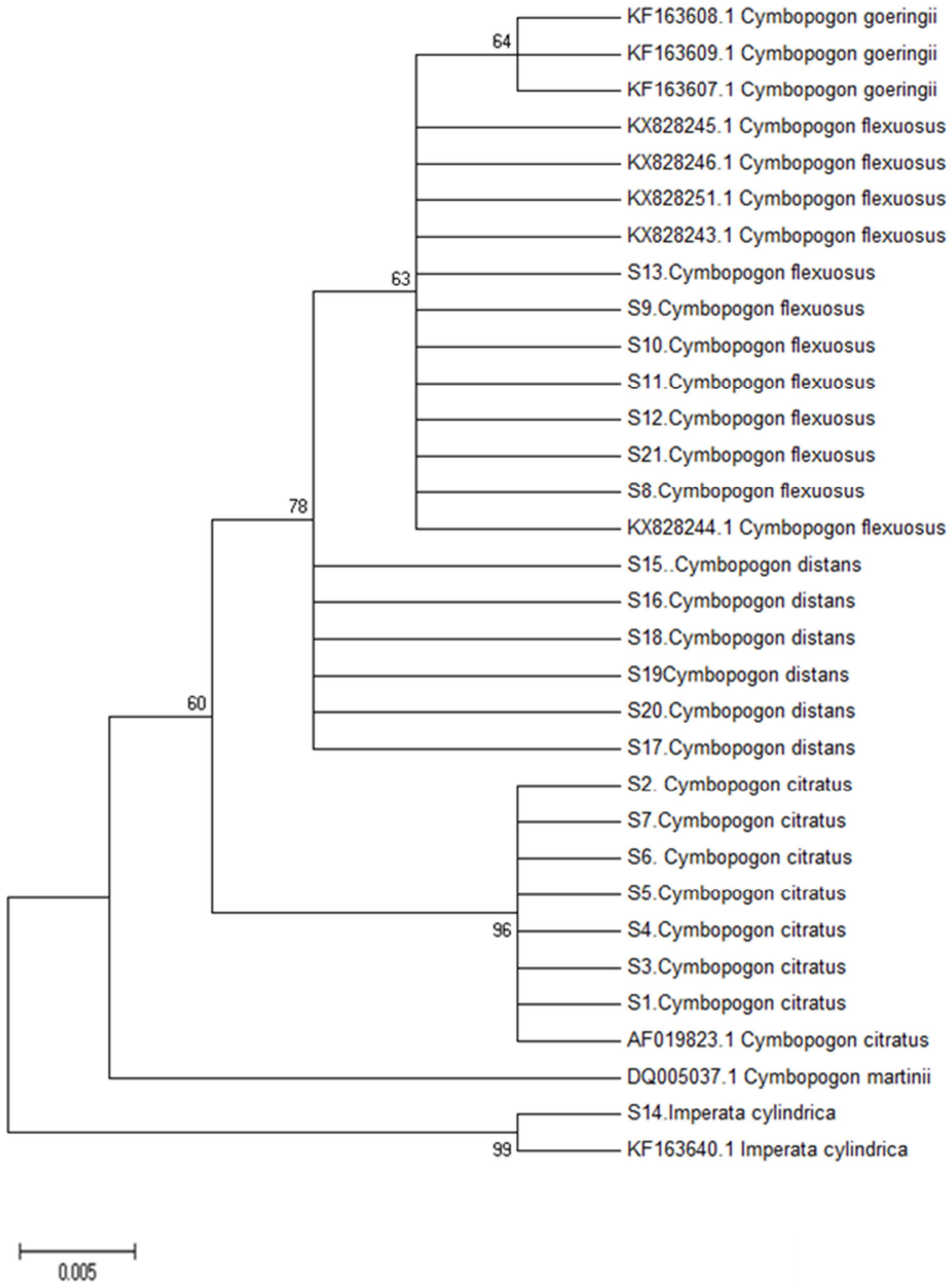
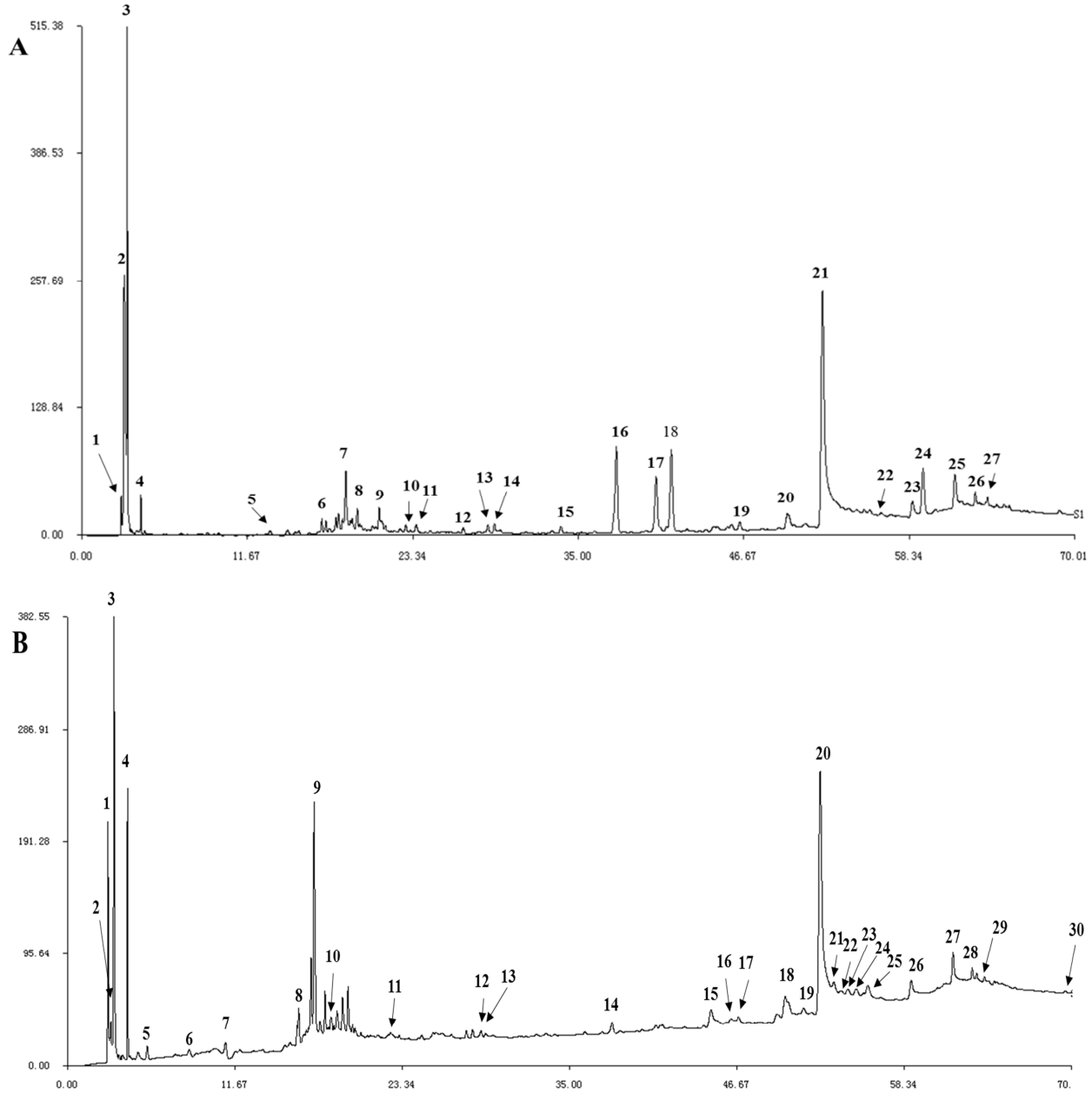


Figure 3. *Phylogenetic tree of the family Poaceae constructed with the ITS2 sequences by using the Neighbor-Joining method.*

3.3. HPLC Fingerprint Analysis

3.3.1. Establishment of Fingerprint Chromatograms for C. distans and Its Adulterants

Twenty tested samples solutions were prepared as described above in the section 3.1, and the samples solutions were submitted to the same chromatography conditions as reported in the section 3.3, and chromatograms of all samples were recorded for 70 minutes. The finding demonstrates that 30, 27 and 21 peaks were common fingerprint among six samples of *C. distans*, seven batches of *C. citratus*, and six bactches of *C. flexuosus*, respectively (Figure 4, 7A, 7B, 7C). In addition, the reference chromatogram fingerprint of *C. distans* showed that the content composition of *C. distans* was quite different from its closely related species (Figure 7A). The total of 16 peaks in *C. distans* were different from its closely related species (Figure 6); and peak No. 1, 5-7, 11-19, 21-24, and No. 30 can be used to discriminate the species of *C. distans* from it closely related species as well as its adulterants and 14 peaks were common between the three references chromatogram of *C. distans*, *C. citratus* and *C. flexuosus*, these common peaks showed the similarity between them and these peaks can be used to classified them into closely related species (Table 9) (Figure 5, 7D)., Furthermore, the data analysis was carried out using the SOP of Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A) which was developed by Chinese Pharmacopoeia Committee, produced reference chromatogram R. Reference chromatogram R was regenerated with the aid of fingerprint chromatogram similarity calculation software.



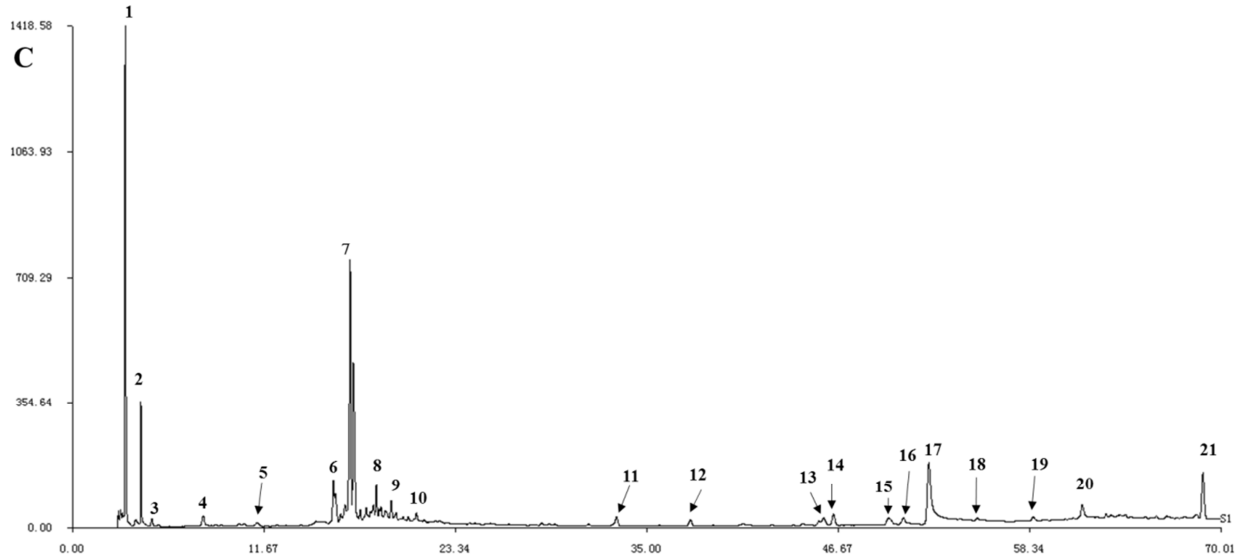


Figure 4. *Reference fingerprint chromatogram of C. citratus (A) 27 commons peaks were identified among six batches of C.citratus, the reference fingerprint chromatogram of C. distans (B)30 common peaks were identified, the reference fingerprint of C. flexuosus (C) 21common peaks were identified.*

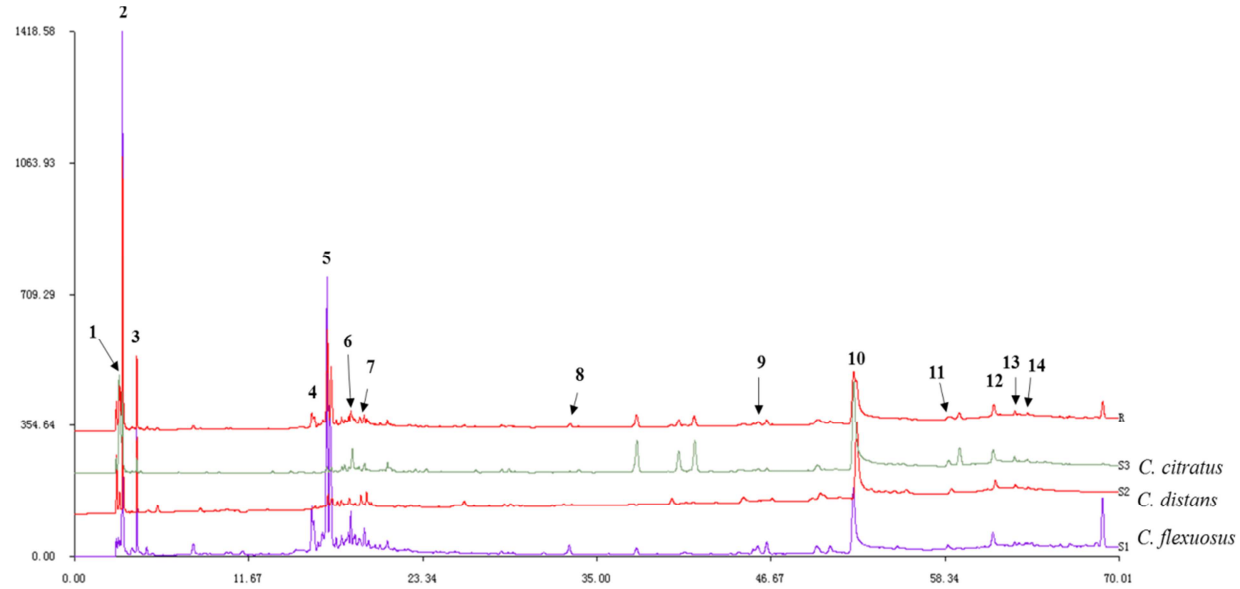


Figure 5. *The reference chromatogram of batches of C. distans, bacthes of C. citratus and batches of C. flexuosus, 14 peaks were common between them.*

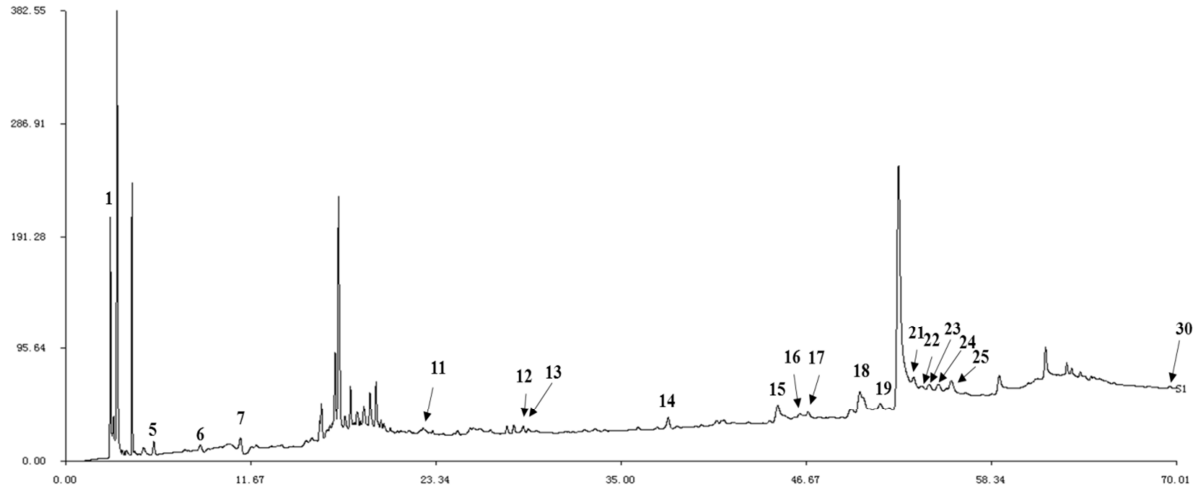


Figure 6. *The reference fingerprint of C. distans indicate the 16 different peaks found in C. distans.*

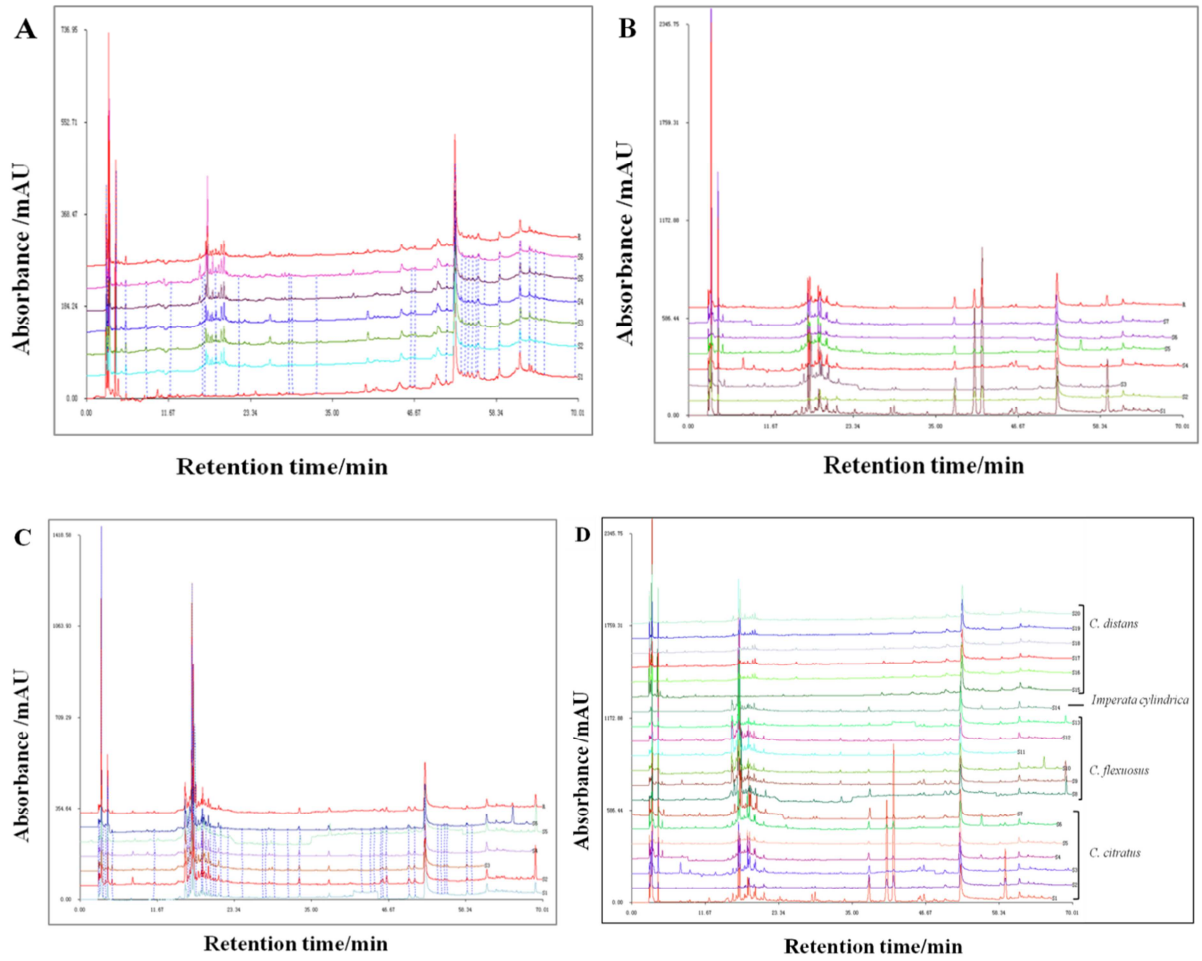


Figure 7. *HPLC fingerprint measured at UV 224 nm of six samples of C. distans (A), seven lots of C. citratus (B), six samples of C. flexuosus (C), and C. distans and its adulterants (D).*

3.3.2. Similarity Assessment

Findings from the fingerprint chromatograms analysis of 7 batches *C. citratus* indicate that the similarity indexes obtained from 27 common peaks were >0.91 for 5 samples and the similarity of 2 samples with batches number S1 and S2 were <0.91(Table 7) (Figure 7B). The fingerprint chromatograms analysis of 6 batches of *C. flexuosus* show that the similarity indexes obtained from 21 common peaks were more than 0.93 for all the samples (Table 8) (Figure 7C). The fingerprint chromatograms analysis of 6 batches *C. distans* displayed that the similarity indexes obtained from 30 common peaks were >0.91 (Table 6) (Figure 7A), except the S15 which had the value of less than 0.91. Finally, the similarity indexes between species of *C. distans*, *C. citratus*, *C. flexuosus* and *Imperata cylindrica* were all less than 0.93 (Table 9). Thus, it could be concluded that, 7 samples of *C. citratus* from different provinces, in which 5 samples originating from Guangdong, Hebei, Anhui and Sichuan shared similar HPLC fingerprints and the internal qualities of the two samples of *C. citratus* S1 and S2 from Yunnan province were unstable. While, the quality of the six samples of *C. flexuosus* from Guangxi, Sichuan, Hebei, Anhui shared similar HPLC fingerprints among them. From the six samples of *C. distans*, one sample S15 collected in Sichuan Province was not stable and the quality of the other 5 samples of *C. distans* collected from different geographical origins shared similar HPLC fingerprints. Finally, the similarity evaluation of the twenty collected samples (Table 9) shows a significant difference between *C. distans* and its adulterants.

Table 6. *The similarity evaluation of C. distans from different provinces.*

No.	S15	S16	S17	S18	S19	S20	Reference
S15	1.000	0.827	0.828	0.834	0.833	0.754	0.890
S16	0.827	1.000	0.997	0.991	0.983	0.962	0.990
S17	0.828	0.997	1.000	0.992	0.977	0.953	0.988
S18	0.834	0.991	0.992	1.000	0.977	0.940	0.986
S19	0.833	0.983	0.977	0.977	1.000	0.968	0.987
S20	0.754	0.962	0.953	0.940	0.968	1.000	0.956
Reference	0.890	0.99	0.988	0.986	0.987	0.956	1.000

Table 7. *Similarity evaluation of 7 lots of C. citratus from different provinces.*

No.	S1	S2	S3	S4	S5	S6	S7	Reference
S1	1.000	0.546	0.499	0.512	0.531	0.486	0.493	0.731
S2	0.546	1.000	0.56	0.792	0.572	0.676	0.492	0.719
S3	0.499	0.560	1.000	0.865	0.953	0.941	0.958	0.930
S4	0.512	0.792	0.865	1.000	0.881	0.916	0.847	0.914
S5	0.531	0.572	0.953	0.881	1.000	0.976	0.989	0.951
S6	0.486	0.676	0.941	0.916	0.976	1.000	0.956	0.943
S7	0.493	0.492	0.958	0.847	0.989	0.956	1.000	0.927
Reference	0.731	0.719	0.930	0.914	0.951	0.943	0.927	1.000

Table 8. *The similarity of the chromatographic patterns obtained from the 6 samples of C. flexuosus.*

No.	S8	S9	S10	S11	S12	S13	Reference
S8	1.000	0.907	0.883	0.893	0.849	0.900	0.934
S9	0.907	1.000	0.951	0.959	0.868	0.931	0.973
S10	0.883	0.951	1.000	0.989	0.856	0.978	0.982
S11	0.893	0.959	0.989	1.000	0.886	0.982	0.990
S12	0.849	0.868	0.856	0.886	1.000	0.875	0.919
S13	0.900	0.931	0.978	0.982	0.875	1.000	0.981
Reference	0.934	0.973	0.982	0.990	0.919	0.981	1.000

Table 9. *The similarity of the chromatographic patterns obtained from the 20 samples from different provinces.*

Sample no.	Similarity	Sample no.	Similarity
S1	0.612	S11	0.741
S2	0.705	S12	0.770
S3	0.903	S13	0.825
S4	0.853	S14	0.620
S5	0.871	S15	0.213
S6	0.858	S16	0.485
S7	0.836	S17	0.474
S8	0.679	S18	0.478
S9	0.868	S19	0.440
S10	0.688	S20	0.539

3.3.3. Hierarchical Clustering Analysis (HCA)

The concepts of dissimilarity and similarity are basic to cluster analysis. HCA was performed with the aid of SPSS software version 16, USA. The peaks areas of all the samples were systematically clustered and the Ward method and Squared of the Euclidean distance were selected as a measure of similarity between samples of different batches. In addition, the reliability and accuracy of chromatographic fingerprints using similarity calculations were further demonstrated by HPLC-based. HCA, a widely used technique for differentiating herbal plant species, exhibited the relationships between the twenty samples collected from various regions (Figure 8). All the twenty samples including six samples of batch of *C. distans*, seven samples of *C. citratus*, one sample of *Imperata cylindrica* and six samples *C. flexuosus* were grouped into three main classes or clusters; the six samples of *C. distans* (S15- S20) were classed into group one, samples of *C. flexuosus* with batch numbers (S9- S13) were classed into the second group and two additional samples with batch number S14 corresponded to *Imperata cylindrica* and one sample of *C. citratus* with batch number S2 were also classed into the second group. The HCA also shows the high similarity between these three species found in the second group. The third class encompasses sample of *C. citratus* with batch number S1, S3, S4, S5, S6, S7 and one sample of *C. flexuosus* with batch number S8. Given the characteristics of the regions, HCA could show significant difference between *C. distans* from its adulterants. Consequently, the obtained results from HCA are consistent with that of the SA.

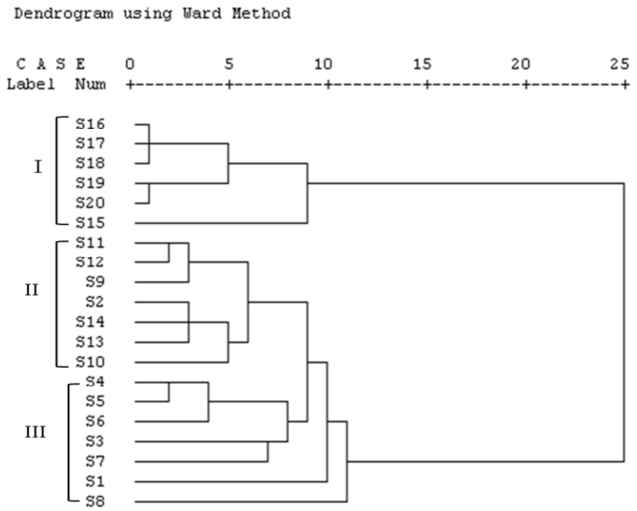


Figure 8. *Dendrogram of cluster analysis of C. distans and its adulterants.*

4. Discussion

In many prior investigations, the identification techniques of medicinal plants such as *C. citratus*, *Imperata cylindrica* and *C. flexuosus* have been mainly focused on characterization of morphology, it is challenging to recognize *C. distans* and its adulterants by experienced or trained experts based on their morphological characteristics. Recently, molecular techniques such as DNA barcoding have been successfully utilized for accurate and rapid identification of

species, diversity and ecological studies [14], with the ITS2 barcode demonstrating noticeable stability and accuracy in this area. ITS2 has been successfully utilized in researches of identifying medicinal species and their closely related species, as result, lots of medicinal species like *Rosaceae*, *Ephedrae herba* and *Corni Fructus* have been effectively identified using ITS2 sequence [26-29]. According to Hou et al. [29], ITS2 region was proven to successfully differentiate *Corni Fructus* from its adulterants. Xin et al. [30] indicated that the ITS2 barcode as a powerful tool for tracing *Goji*. Their results also showed that this technique precisely identify *Ephedrae herba* and their closely related species [27]. The ITS2 region has also been used by other authors for identification, for example, for spider mites [31], for *sycophila* [32], for *Corydalis boweri* [33], *Meconopsis horridula* and their closely related species of the same genus, and for Fasciola [34]. Furthermore, ITS1 was used to reveal that species of *Amomum villosum* belongs to the family Zingiberaceae [35].

In contrast to other reports, this investigation provides a strong case for the combination of microscopic evaluation, HPLC fingerprint and ITS2 region being the most promising universal techniques for validating *C. distans* and their closely related species, and adulterants. In molecular identification, despite the fact that plants share many morphological similarities, ITS2 region was found not only able to differentiate plant taxa from diverse plant species but also capable to discriminate closely related taxa at the genus and species stages, suggesting that ITS2 as DNA barcorde in plants is a suitable technology for distinguishing the species *C. distans*, even though ITS2 has been extensively used to distinguish the medicinal species. These findings were in consistent with above studies who also demonstrated variation for closely-related species. This study obviously demonstrates that DNA barcoding using candidate like ITS2 is a reliable method for differentiating *C. distans* from the other plant species, which can also be applied to rapid identification of medicinal plants and their adulterants or substitutes.

Previous studies have reported that microscopic assessment to be helpful for the quality control of plant species including *C. citratus* and discrimination from drugs and adulterants [36]. E.L. Kotina et al. [37] studied the anatomical characteristics of leaves of *Warburgia salutaris* (Canellaceae). Eltahir and al. [38] demonstrated that the number of spongy parenchyma cells and the quantities of oil detected in the leaf epidermal cells of *C. citratus* could make the difference between *C. citratus* and *Cymbopogon schoenanthus* and avoid adulteration. In this study, the microscopic assessment of *C. distans* showed that the spongy parenchyma is constituted of 1-2 layers in *C. citratus* followed by the upper epidermis, while the lower epidermis in *C. distans* is constituted of one layer of small cell following by the spongy mesophyll that is located between vascular bunbles.

Finally, the fingerprint chromatograms of 6 lots of *C. distans* collected from different provinces showed that the similarity between all species were all more than 0.91 except S15 collected in Sichuan Province, which was affected by local ecological environmental factors and the fingerprint chromatograms of 6 batches of *C. flexuous* from different provinces indicated that all samples were all more than 0.91 and the result showed that chemical composition of *C. flexuous* collected in different province shared similar HPLC fingerprints. In addition, the similarity of 7 batches of *C. citratus* showed that 5 samples were all more than 0.91 and two samples S1 and S2 were less than 09 indicating that the samples were also unstable. 14 peaks were common between *C. distans*, *C. citratus*, *C. flexuous* and *Imperata cylindrica* and the similarity were all less than 0.93. The result showed the great differences between *C. distans* and its adulterants.

5. Conclusions

In conclusion, molecular, chemical fingerprint and microscopic analysis were used to evaluate the authentication, quality and safety of *C. distans* from its adulterants. ITS2 was found to be the suitable barcode for identification and could discriminate the *C. distans* species from its adulterants. For microscopic analysis, both the morphology and anatomy structures could distinguish *C. distans* species based on the measurement given in Flora of China and can also be used for screening *C. distans* and its adulterants and closely related species. The study also showed that HPLC fingerprint of 6 batches of *C. distans*, 7 batches of *C. citratus*, and 6 batches of *C. flexuosus* from different regions were successfully assessed by similarity evaluation and HCA methods. For chemical fingerprint analysis, 30 peaks of all the 6 batches of *C. distans* were assigned as “common peaks”, and 27 peaks were common among the 7 batches of *C. citratus*, 21 common peaks were found among 6 batches of *C. flexuosus* and the similarities were all more than 0.93, indicating that the samples from different geographical origins shared similar HPLC fingerprints. In addition, the chromatogram fingerprint of the twenty collected samples showed the significance difference between *C. distans* and its closely related species,14 common peaks was found between them; and the similarities were all less than 0.93, demonstrating that HLPC fingerprint was found to be effective in assessing *C. distans* from it adulterant and governing the quality of *C. distans*. Finally, these results indicated that analytical method developed in the current work can be simple and powerful techniques that provide full-scale qualitative and quantitative data for assessing the quality of *C. distans*, and it can also be a valuable reference for the further research and development of this species and its pharmaceutical products.

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