Identification of *Clematidis radix* et Rhizoma and its adulterants by core haplotype based on the ITS sequences

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ABSTRACT. To develop a method to identify *Clematidis radix* et Rhizoma using sequence similarity and sequence-specific genetic polymorphisms based on the ITS sequences. DNA was extracted from leaves of *Clematis mandshurica* Rupr and *C. hexapetala* using a DNA extraction kit. ITS sequences were amplified by PCR, and analyzed in Contig Express, DNAman, and MEGA 5.0. The core haplotype was determined, and similarities between the core and other haplotypes were calculated. In total, 138 ITS sequences of *C. mandshurica* were obtained with a length of 611 bp. The similarity threshold between *C. mandshurica* and counterfeit species was 99%. Using specific mutation sites, we could identify *C. chinensis*, *C. hexapetala*, and *C. mandshurica* rapidly and accurately. A new DNA-based method has been established to rapidly and accurately identify *Clematidis radix* et Rhizoma.

Key words: *Clematidis radix* et Rhizoma; Core haplotype; Identification threshold; Mutation sites

INTRODUCTION

Clematidis radix et Rhizoma is the dry radix and rhizome of *Clematis chinensis* Osbeck, *C. hexapetala* Pall, and *C. mandshurica*, (Rupr Zhou Y, et al. 2012). In traditional Chinese medicine, *Radix et Rhizoma Clematidis* is applied to dispel wind dampness, flush the meridian, and treat rheumatic paralysis, limb numbness, and tendon spasm. Recent pharmacological studies have also shown that *Clematidis radix et Rhizoma* has anti-

inflammatory, hypoglycemic, anti-hypertensive, and anti-tumor activities (Zhou Y, et al., 2012, Han W et al., 2013, Ionkova I 2011, Jung Up Park JN, et al. 2016, Mitjans M, et al. 2005), Yang J, et al. 2017). Because of wide application in clinics, adulterants of *Clematidis radix et Rhizoma* have proliferated in the market, including *C. armandii* Franch., *C. finetiana* H. Lév. et Vaniot, *C. uncinata* Champ., *C. henryi* Oliv., *C. florida* Thunb., *C. chrysocoma* Franch., *C. lasiandra* Maxim, *C. peterae* Hand.-Mazz., *C. kerriana* J.R. Drumm. et Craib, and *C. leschenaultiana* DC . Huang YY (2005), Song L et al. (2011), Li JS et al. (1980). These adulterants are traditionally identified based on the practitioner's experience, variations in morphology due to the age of herbs and environmental conditions, and other factors. However, such factors are inadequate for accurate identification, because *Clematidis radix et Rhizoma* cannot be distinguished from adulterants morphologically or microscopically. However, genuine *Clematidis* and adulterants differ greatly in chemical composition and efficacy, and improper application may lead to drug safety issues. Song L, et al. (2011), Li JS et al., (1980), Guo LX et al., (2015). Therefore, it is of considerable importance to establish a rapid, accurate, and efficient method to identify genuine *Clematidis*.

DNA barcoding for species identification was first proposed by the Canadian zoologist Hebert in 2003. (Hebert PDN et al., 2003). This molecular diagnostic technology is based on short sequence fragments, and has been used since then to identify a variety of herbal medicines rapidly, efficiently, accurately, and objectively. (Yan HX et al., 2010, Hou DY et al., 2013, Chen JJ et al., 2015). Current methods of DNA barcoding include similarity searching, distance calculations, and tree-building. Similarity searching is mainly based on the BLAST algorithm. (Chen SL, et al., 2013, Cheng XL, et al., 2012 and Liu J, et al., 2011). However, due to the lack of a well-defined identification threshold, and frequently changing reference sequences, standardized identification is yet to be established for many herbal medicines. Consequently, a sample may be misidentified because a BLAST search may return two or more species with 99% similarity, or a neighbour-joining tree may cluster different species into a single branch. In addition, sequences from some species may not be present in existing databases, and are thus challenging to identify.

One way to identify a plant is to determine its haplotype, which typically consists of a DNA fragment with a number of nucleotide polymorphisms. The most frequent haplotype found within a species is considered the core haplotype, and all others are deemed rare. As would be expected, the similarity between the core and a rare haplotype of the same species is larger than the similarity between the core haplotype and a adulterant of the same genus. Therefore, a robust, well-defined similarity threshold value based on a large number of sequences could improve the accuracy, objectivity, and speed of DNA-based identification. In this study, we constructed a library of ITS sequences using 138 samples of *C. mandshurica* Rupr to determine the core haplotype, set the identification threshold value, and identify species-specific nucleotide variants, if any. Based on these parameters, we were able to identify samples of *C. hexapetala*, as well as *C. chinensis* and adulterant herbs from GenBank. Thus, we have established a rapid, accurate, and efficient molecular method to identify *Clematidis radix et Rhizoma.*

MATERIAL AND METHODS

Plant materials

Leaves of *C. mandshurica* and *C. hexapetala* were collected from Liaoning Province, Heilongjiang Province, and Inner Mongolia Autonomous Region in China (Tables 1 and 2). ITS sequences from *C. chinensis* and adulterant herbs were downloaded from GenBank (Table 3).

DNA extraction and PCR

Total DNA was extracted from approximately 0.1 g fresh leaves using a plant DNA extraction kit (Beijing Biomed Co., Ltd., Beijing, China), following the manufacturers protocol. Final DNA concentration was determined by spectrophotometry, and integrity was examined by electrophoresis on 1% (w/v) agarose. The universal primers ITS-F (5′-AGAAGTCGTAACAAGGTTTCCGTAGG-3′) and ITS-R (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify full length ITS sequences with the following program: Initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 1 min; and

final extension at 72°C for 10 min. Amplified fragments were sequenced by Sangon Biotechnology Co, Ltd. (Shanghai, China).

Table 1. Voucher data for samples of *Clematis mandshurica* Rupr.

No.	Origin	Altitude (m)	Longitude	Latitude
1	Dalianzhuanghe, Liaoning Province	43	N31°41′40″	E123°2′14″
2	Fengcheng, Liaoning Province	219	N40°46′ 55″	E123°54′9″
3	Hengren, Liaoning Province	309	N41°14′15″	E125°22′12″
4	Dengta, Liaoning Province	173	N41°22′1″	E123°31′55″
5	Kuandian, Liaoning Province	310	N40°58′35″	E125°0′13″
6	Benxi, Liaoning Province	247	N41°18′0″	E124°6′25″
7	Qingyuan, Fushun, Liaoning Province	387	N42°6′58″	E124°55′39″
8	Youyan, Anshan, Liaoning Province	40	N40°15′51″	E123°13′41″
9	Dandong, Liaoning Province	204	N39°1′34″	E129°15′41″
10	Qianshan, Liaoning Province	129	N41°1′37″	E123°8′22″
11	Kaiyuan, Liaoning Province	98	N42°22′43″	E124°0′47″
12	Tieling, Liaoning Province	118	N42°8′38″	E123°43′53″
13	Xifeng, Liaoning Province	265	N42°45′26″	E124°43′21″
14	Faku, Liaoning Province	211	N42°27′59″	E123°11′59″
15	Liaoyang, Liaoning Province	248	N42°56′12″	E123°22′27″
16	Xinbin, Liaoning Province	258	N41°47′12″	E124°38′20″
17	Fusong, Baishan, Jilin Province	522	N42°20′35″	E127°15′48″
18	Dongliao, Jilin Province	423	N42°48′44″	E124°57′13″
19	Lishu, Jilin Province	255	N46°0′32″	E130°40′6″
20	Yanji, Jilin Province	160	N42°55′20″	E129°35′24″
21	Liuhe, Jilin Province	445	N42°16′8″	E125°44′40″
22	Ji′an, Tonghua, Jilin Province	309	N41°10′40″	E126°16′10″
23	Tonghe, Ha′erbin, Heilongjiang Province	116	N46°16′53″	E129°20′43″
24	Nancha, Yichun, Heilongjiang Province	153	N47°6′47″	E129°22′20″
25	Jiamusi, Heilongjiang Province	102	N46°45′16″	E130°22′38″
26	Tangyuan, Heilongjiang Province	152	N46°40′1″	E129°37′32″
27	Muleng, Mudanjiang, Heilongjiang Province	297	N44°53′7″	E130°33′33″
28	Hailin, Mudanjiang, Heilongjiang Province	265	N45°10′49″	E129°24′10″
29	Yilan, Heilongjiang Province	109	N46°20′53″	E129°34′8″
30	Shuangyashan, Heilongjiang Province	450	N46°22′8″	E131°6′6″

Table 2. Voucher data for samples of *C. hexapetala* Pall.

No.	Origin	Altitude (m)	Longitude	Latitude
1	Nianzishan, Heilongjiang Province	398	N47°32′44″	E122°51′17″
2	Xiaoyingxiang, Yanji, Jilin Province	160	N42°55′20″	E129°35′24″
3	Dalianzhuanghe, Liaoning Province	43	N31°41′40″	E123°2′13″
4	Horqin Right Wing Front Banner, Inner Mongolia	541	N46°37′15″	E121°9′24″
5	Faku, Liaoning Province	211	N42°27′59″	E123°11′59″
6	Muleng, Heilongjiang Province	297	N44°53′7″	E130°33′33″

Table 3. GenBank accession numbers for ITS sequences of *Clematis chinensis* Osbeck and counterfeits of *Clematidis radix* et Rhizoma.

Var.	Accession numbers			
Clematis chinensis	AB775161.1	AB775169.1	AB775162.1	AB775170.1
	AB775163.1	AB775171.1	AB775164.1	AB775172.1
	AB775165.1	AB775173.1	AB775166.1	AB775174.1
	AB775167.1	GU732584.1	AB775168.1	JF714641.1
Clematis armandii	FJ572047.1			
Clematis chrysocoma	GU732585.1	GU732587.1	GU732586.1	
Clematis finetiana	GU732593.1	JF714642.1		
Clematis florida	KC004031.1			
Clematis henryi	JF714645.1			
Clematis kirilowii	KC758681.1			
Clematis lasiandra	GU732600.1	JF714640.1		
Clematis leschenaultiana	GU732603.1			
Clematis peterae	GU732614.1	GU732615.1		
Clematis pogonandra	GU732618.1			
Clematis uncinata	GU732637.1	JF714643.1		

Sequence proof-reading and statistical analysis

Sequences were manually proofread in ContigExpress (Vector NTI, Invitrogen, Carlsbad, CA, USA), and aligned using blastn (available at BLAST NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Species-specific nucleotide variants were identified with DNAman (Lynnon Biosoft, San Ramon, CA, USA) and MEGA-5.0. The core haplotype was determined, and similarities between the core and other haplotypes were calculated to set the identification threshold value.

RESULTS

Amplification of ITS sequences

Electrophoresis confirmed that PCR products were of the expected length, highly pure and suitable for further analysis (Figure 1).

Figure 1. DNA fragments amplified by PCR. Lane M, DL2000 DNA marker; Lanes 1–5, amplified ITS fragments.

Sequence analysis

We obtained 138 ITS sequences of *C. mandshurica* Rupr from 21 locations. The length of the aligned matrix was 611 bp, with 50 polymorphic sites and 86 different haplotypes. After removing haplotypes due to hybridization, 8 distinct haplotypes were found. Polymorphic sites are listed in Table 4, and haplotype frequency is summarized in Figure 2.

Table 4. Polymorphic sites in *Clematis mandshurica* Rupr. haplotypes.

Haplotypes	Polymorphic sites (bp)										
	4	5	6	19	26	55	94	150	428	566	591
H1	A	C	C	A	A	A	C	G	C	C	C
H2	*	-	-	*	*	*	T	*	*	T	G
H3	*	*	*	*	*	*	T	*	*	T	G
H4	-	-	-	-	-	*	*	*	*	T	G
H5	*	*	*	*	*	*	T	*	*	*	G
H6	*	*	*	*	*	T	*	*	*	*	G
H7	*	*	*	*	*	T	T	A	T	T	G
H8	*	*	*	*	*	*	*	*	*	T	G

(Note: *indicates identity to H1; - indicates deletion).

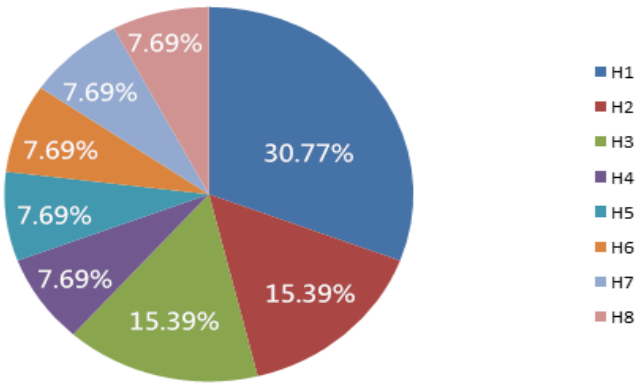

Figure 2. Frequency of *Clematis mandshurica* Rupr. haplotypes.

H1 was the most frequent haplotype at 30.77%, and was therefore considered the core haplotype for *C. mandshurica* Rupr. Accordingly, all other haplotypes were deemed rare. The similarity between the core and rare haplotypes was 99.35–100% based on BLAST alignment. On the other hand, the similarity between the core haplotype and the ITS fragment in *C. hexapetala* and *C. chinensis* was 97.23–98.85% and 97.46–98.73%, respectively. Furthermore, the similarity between the core haplotype and ITS sequences in adulterant herbs of the same genus was 93.07–97.86%. Therefore, 99% sequence similarity to the core haplotype was considered adequate to identify genuine *C. mandshurica* Rupr. DNA man inspection of ITS sequences in *C. chinensis*, *C. hexapetala*, *C. mandshurica*, and adulterants identified species-specific polymorphisms (Table 5). In particular, there was a clear distinction between adulterants and *Radix et Rhizoma Clematidis* at nucleotides 35, 86–89, 92, 159, 166, 422 and 555. In *C. hexapetala*, the bases at sites 89, 94, 95, 98, 158, 166, and 174 were G, G, A, T, G, T, and G, respectively, which significantly separated this species from *C. chinensis* and *C. mandshurica*. Finally, nucleotides 89, 98, and 570 were A, T, and T in *C. mandshurica*, but C, deletion, and C in *C. chinensis*, clearly separating the two species.

Table 5. Polymorphic sites in haplotypes of *Clematis chinensis* Osbeck, *C. hexapetala* Pall., *C. mandshurica* Rupr., and

Species.	Polymorphic sites (bp)																			
	35	86	87	88	89	92	94	95	98	158	159	165	166	174	193	422	555	565	566	570
C. mandshurica	C	T	T	A	A	G	T	C	C	A	A	G	C	A	C	C	C	G	G	T
	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	C
	*	*	*	*	*	*	*	*	T	*	*	*	T	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
C. hexapetala	*	*	*	*	G	*	G	A	T	G	*	*	T	G	*	*	*	*	*	*
	*	*	*	*	G	*	G	A	T	G	*	*	T	G	*	*	*	*	*	C
	*	*	*	*	G	*	G	A	T	G	*	*	T	G	*	*	*	*	*	*
C. chinensis	*	C	C	G	-	T	-	*	*	*	*	T	*	*	G	*	*	*	*	C
	*	*	*	G	-	T	-	*	*	*	*	T	*	*	G	*	*	*	*	C
	*	C	*	G	-	T	-	*	*	*	*	T	*	*	G	*	*	*	*	C
	*	*	*	-	-	*	*	*	*	*	*	T	*	*	G	*	*	*	*	C
	T	C	C	-	-	C	*	*	*	*	*	*	T	*	*	*	*	*	*	C
	*	C	*	G	-	T	-	*	*	*	*	T	*	*	G	*	*	*	*	C
	*	*	*	G	-	T	*	*	*	*	*	*	*	G	G	*	*	*	*	C
	*	C	*	-	-	*	*	*	T	*	*	*	*	*	G	*	*	*	*	C
C. henryi	T	*	C	G	C	*	C	T	T	-	-	-	-	*	T	A	T	-	-	*
C. finetiana	T	C	-	-		*	C	*	*	*	C	*	T	*	*	A	T	-	-	
C. finetiana	T	C	-	-	-	*	C	*	*	*	C	*	T	*	*	A	T	-	-	-
C. chrysocoma	T	-	-	-	-	A	A	A	*	G	C	T	T	*	T	A	T	-	-	*
C. lasiandra	T	*	C	G	C	*	C	T	*	*	C	*	T	*	T	A	T	-	-	*
C. kirilowii	T	C	C	G	-	T	-	*	*	G	*	T	*	*	G	*	*	*	*	*
C. leschenaultiana	T	*	C	G	C	*	C	T	T	*	C	*	T	*	T	A	T	-	-	*
C. peerage	T	*	C	G	C	*	C	*	*	*	C	*	T	*	T	A	*	-	-	*
C. pogonandra	T	*	C	G	C	*	C	*	T	C	C	*	T	*	T	A	T	-	-	*
C. uncinata	T	C	-	-	-	*	*	*	*	*	C	*	T	*	*	A	T	-	-	C
C. armandii	T	*	C	G	T	*	C	*	-	*	C	*	T	*	T	A	*	-	-	*

(Note : *indicates identity to the first sample; - indicates nucleotide deletion. Species-specific variants in *C. mandshurica*, *C. hexapetala*, *C. chinensis*, and counterfeits are highlighted in red, blue, green, and purple, respectively).

DISCUSSION AND CONCLUSION

Yang successfully used random amplified polymorphic DNA to assess the quality of *Radix et Rhizoma Clematidis* for 11 taxa of *Clematis*, but this method is poorly reproducible. On the other hand, PCR-single-strand conformation polymorphism was used to trace the origin of *Clematis* samples, but this technology is relatively cumbersome and time-consuming. Thus, ITS, ITS2, and psbA are commonly used as DNA barcodes for *Radix et Rhizoma Clematidis*. However, the accuracy, objectivity, and speed of identification based on these sequences are unsatisfactory, because comparison of a large number of sequences is required, along with an identification standard. In this study, we established a similarity threshold value for identifying *C. mandshurica* and adulterants. In addition, we found that *Clematidis radix et Rhizoma* could be identified accurately based on specific nucleotide sites in ITS sequences.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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