academicJournals

Vol. 11(23), pp. 373-379, 17 June, 2017
DOI: 10.5897/JMPR2017.6408
Article Number: 929A4EB64725
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Identification of Campsis Flos and its common adulterants using ITS2 sequence of DNA molecular barcoding

Ye Lu[1]*, Baoling Ju[2], Liang Gao[1] and Jin Wang[1]

[1]College of Pharmaceutical Science, Soochow University, Jiangsu Suzhou 215123, China.
[2]Mudan Jiang Medical University, Heilongjiang, Mudan Jiang 157011, China.

Received 28 April, 2017; Accepted 30 May, 2017

Internal transcribed spacer 2 (ITS2) is known as a good sequence of DNA molecular barcoding. In this study, the authors authenticated Campsis Flos and its common adulterants based on ITS2 sequence. Campsis Flos has been used in traditional Chinese medicine for many years as a blood activator to promote menstruation. Among its adulterants in the market, the most common are the flowers of *Paulownia tomentosa*, *Hibiscus syriacus* and *Rhododendron molle*. To discriminate Campsis Flos from these adulterants and control its quality, safety and efficacy, the total genomic DNA was extracted from the leaves of *Campsis grandiflora*, *Campsis radicans*, and their common adulterants. The internal transcribed spacer 2 (ITS2) of ribosomal DNA was sequenced after PCR amplifying. A neighbor-joining (NJ) tree was constructed using MEGA 6.0. The ITS2 secondary structure was predicted by an ITS2 web server. The results showed that the ITS2 sequence lengths of *C. grandiflora*, *C. radicans*, and *P. tomentosa* were 246–248, 246, and 229–233 bp, respectively. The ITS2 sequence lengths of *H. syriacus*, *H. syriacus* L. f. var. *syriacus* f. *amplissimus*, and *R. molle* were all 230 bp. The ITS2 secondary structure of Campsis Flos was then effectively distinguished from its adulterants. In conclusion, barcode ITS2 sequence could be used to rapidly and accurately identify Campsis Flos from its adulterants to promote quality control and standardization.

Key words: DNA molecular barcoding, ITS2 sequence, Campsis Flos, identification.

INTRODUCTION

Campsis Flos is made from the dried flowers of *Campsis grandiflora* and *Campsis radicans* and is used in traditional Chinese medicine. Chinese Pharmacopoeia states that Campsis Flos activates blood to promote menstruation (Commission of Chinese Pharmacopoeia 2015). Recently, researchers have found antidepressive (Yu et al., 2015), antioxidative and anti-inflammatory activities (Cui et al. 2006) in Campsis Flos extract. Campsis Flos is not expensive, but there are some adulterants in the market (He et al., 2007). The common adulterants are the flowers of *Paulownia tomentosa, Hibiscus syriacus* and *Rhododendron molle*. Misuse will

*Corresponding author. E-mail: t_luye@suda.edu.cn. Tel: +86 512 6588 2090. Fax: +86 512 65882089.

will produce side effects, especially because *R. molle* flowers are toxic. Therefore, identifying Campsis Flos is very important. Traditional microscopic identification is too specialized and only a few people can know it (He et al., 2007). To solve this problem, there is need to find a simple and rapid molecular identification method to distinguish the Campsis Flos from its common adulterants.

DNA molecular barcoding has become a potential identification tool for plants and animals (Kress et al., 2005, 2007; Zhang et al., 2015; Chen et al., 2013). This method can be used to promote quality control and standardization in traditional Chinese medicine, and it has been promulgated by the Chinese Pharmacopoeia.

Among DNA barcoding candidate sequences of the chloroplast genes psbA-trnH, matK, rbcL and nuclear ribosomal DNA ITS (internal transcribed spacer) 2, ITS2 is known as a good sequence of identification (Yao et al., 2010; Pang et al., 2013). Chen et al. (2010) also determined the effectiveness of the ITS2 in identifying medicinal plants using samples from a wide range of taxa. Zhang et al. (2016) also recommended ITS2 for use as a mini-barcode. In this study, total genomic DNA was isolated from *C. grandiflora* and *C. radicans* and their adulterants. The ITS2 was amplified by PCR and sequenced. A neighbor-joining (NJ) tree was constructed using MEGA 6.0 software. The maximum genetic distance within species was far less than the minimum genetic distance between species. The Campsis Flos samples were clustered as a class in the NJ tree, and different species were separated. The NJ tree revealed a close genetic relationship between *P. tomentosa and* Campsis Flos; *R. molle* has a closer relationship with Campsis Flos, whereas *H. syriacus* had a farther relationship. The ITS2 secondary structure was predicted by ITS2 web server. ITS2 secondary structures of the different species were significantly different from each other, whereas those of the same species had no significant differences. Thus, ITS2 sequence offers a new alternative for rapid and straightforward identification of Campsis Flos and its common adulterants.

MATERIALS AND METHODS

Leaf samples of Campsis Flos and its adulterants were collected from Suzhou, Hangzhou, and Enshi in Hubei province. Professor Chunyu Liu from Soochow University identified the species. All voucher specimens were deposited in the herbarium of the College of Pharmaceutical Science of Soochow University. Fresh leaves were dried using silica gel until DNA extraction. Table 1 shows the samples used in this study and their origins, time and place of collection.

DNA extraction, PCR amplification and sequencing

Samples of approximately 50 mg were pulverized using liquid nitrogen and 2% polyvinylpyrrolidone (PVP) with a pestle and mortar. Total genomic DNA was subsequently extracted using the Plant Genomic DNA Kit (CWBiotech Co., Ltd., Beijing, China) following the recommended protocol. The extracted DNA concentrations of all samples were determined using a Q3000-type micro ultraviolet spectrophotometer (Bio-Rad Company, Hercules, CA, USA), and optical density values at 230, 260, and 280 nm of each DNA sample was determined. One pair of universal primers, up (5'-AACCATCGAGTCTTTGAACGC-3') and down (5'-CCTTGTAAGTTTCTTTTCCTCC-3'), was designed for PCR amplification of the ITS2 sequence of Campsis Flos and its adulterants. PCR amplification was carried out in My Cycler (BioRad Lab Inc., USA). PCR was performed using 50 µl reaction mixtures containing 25 µl of 2 × Taq Master Mix (Sangon Biotech Co., Ltd., Shanghai, China), 1 µl of each PCR primer (10 µM), and 0.5 to 1 µl of DNA extract, and the total volume was adjusted to 50 µl with sterile deionized water. PCR amplification was conducted according to the following procedure: 94°C for 3 min, 30 cycles of 94°C for 50 s, 54°C for 50 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis in a 1% agarose gel in 0.5 × TAE buffer with 0.5 µg/ml Gel Red at a constant voltage of 100 V for 40 min. To estimate the size of separated fragments, DNA ladder was loaded into the first lane of each gel. The PCR products were sequenced bidirectionally using an ABI 3730XL sequencer (Sangon Biotech Co., Ltd., Shanghai, China).

Sequence alignment and phylogenetic analysis

The ITS was located in the middle of 18S and 28S of nuclear ribosomal RNA (nrRNA), and the middle part was divided into ITS1 and ITS2 by 5.8S. The 5.8S and 28S sections of the obtained and GenBank sequences were removed using a Hidden Markov model (HMM) (Keller et al., 2009), leaving the ITS2 sequence. All ITS2 sequences were analyzed using MEGA 6.0 (Tamura et al., 2013). An NJ tree (Saitou et al., 1987) was constructed via bootstrap method that was conducted with 1000 replicates; the substitution model was Kimura-2-parameter (K2P) (Kimura, 1980); and gaps were treated as missing data (complete deletion). Intra- and inter-species sequence divergences were also calculated using the K2P model, and gaps were treated as missing data. The ITS2 secondary structure was predicted via the ITS2 database (http://www.its2.bioapps.biozentrum.uni-wuerzburg.de/) (Koetschan et al., 2010).

RESULTS

DNA quality

Table 2 shows the qualities of the extracted DNA. The quality of DNA extracted from the flowers is low. Therefore, DNA was extracted from leaves instead. The results indicated that the leaf DNA was of sufficient quality for subsequent experiments.

ITS2 sequence analysis and intra- and inter-species variations

The average G-C content and length of ITS2 of all samples are listed and aligned, respectively, using the MUSCLE method in Table 3. The *C. grandiflora* from Enshi, when compared with Genbank, had 17 inter-species variable sites (Table 4).

Table 1. Plant samples used in present study.

Samples(species)	Locality of voucher	Code No.	Collection time	GenBank accession number
Campsis grandiflora(Thunb.) Schum	Garden Hotel Suzhou	1	2015.6.5	KX663336
	Enshi in Hubei	2	2015.7.21	
Campsis radicans(L.) Seem.	Shuifang Road,Suzhou	3	2015.4.13	KX663335
Paulownia tomentosa (Thunb.) Steud.	Main Campus, Soochow University	4	2015.4.14	KX663337
	Suzhou International Foreign Language School	5	2015.4.21	
Rhododendron molle(Blume) G. Don	Hangzhou Botanic Garden	6	2015.4.25	KX663338
Hibiscus syriacus Linn.	Suzhou Baitang Ecological Botanic Garden	7	2015.9.6	KX663340
Hibiscus syriacus L. f. var. *syriacus* f. *amplissimus* Gagnep. f.	Suzhou Baitang Ecological Botanic Garden	8	2015.9.6	KX663339
	Yangcheng Lake Campus, Soochow University;	9	2015.4.21	
	Suzhou Jinji Lake Avenue	10	2015.4.13	
Campsis grandiflora(Thunb.) Schum	GenBank	11	-	KJ419313
Campsis radicans(L.) Seem.	GenBank	12	-	KJ419321
Paulownia tomentosa(Thunb.) Steud.	GenBank	13	-	AF478941
Rhododendron molle (Blume) G. *Don*	GenBank	14	-	AF072486
Hibiscus syriacus Linn.	GenBank	15	-	AF460188

Table 2. DNA quality and concentration of Campsis Flos and its adulterants.

Samples	A260/280	Concentration (ng/μL)
Campsis grandiflora	1.98 (Garden Hotel Suzhou)	60.7
	1.77 (Enshi in Hubei)	47.3
Campsis radicans	1.79	398.3
Paulownia tomentosa	1.71 (Main Campus, Soochow University)	988.5
	1.82 (Suzhou International Foreign Language School)	
Rhododendron molle	1.88	24.1
Hibiscus syriacus	1.61	104.7
Hibiscus syriacus Linn. f. var. *syriacus* f. *amplissimus*	1.7 (Suzhou Baitang Ecological Botanic Garden)	61.2
	1.63 (Yangcheng Lake Campus, Soochow University)	239.8
	1.84 (Suzhou Jinji Lake Avenue)	1443.7

The evolution speed of ITS is fast. Nucleotide analysis of the ITS2 sequence could provide more information on inter- and intra-species divergences. The average intraspecific genetic distances calculated by the Kimura-2-parameter model (Kimura, 1980) for Campsis Flos were 0.01375. The interspecific diversities ranged from 0 to 0.055 between *C. grandiflora* from Genbank, Garden Hotel Suzhou, and from Enshi; from 0.005 to 0.010

Table 3. The average G-C content and length of the ITS2 sequence of Campsis Flos and its adulterants.

Species	GC (%)	Length (bp)
Campsis grandiflora (Garden Hotel Suzhou)	76.5	246
Campsis grandiflora (Enshi)	77.4	248
Campsis radicans	76.1	246
Paulownia tomentosa (Main Campus, Soochow University)	73.4	229
Paulownia tomentosa (Suzhou International Foreign Language School)	71.3	233
Rhododendron molle	58.7	230
Hibiscus syriacus	58.3	230
Hibiscus syriacus Linn. f. var. *syriacus* f. *amplissimus* (Suzhou Baitang Ecological Botanic Garden)	57.8	230
Hibiscus syriacus Linn. f. var. *syriacus* f. *amplissimus* (Yangcheng Lake Campus, Soochow University)	59.5	230
Hibiscus syriacus Linn. f. var. *syriacus* f. *amplissimus* (Suzhou Jinji Lake Avenue)	59.5	230

between *H. syriacus* and *H. syriacus* L. f. var. *syriacus* f. *amplissimus* from Baitang Ecological Botanic Garden, Yangcheng Lake Campus, and Jinji Lake Avenue in Suzhou; and from 0.040 to 0.82 between *P. tomentosa* from Main Campus of Soochow University and Suzhou International Foreign Language School (Table 5).

In this study, the minimum genetic distances of intra-species of samples (0.220) were greater than the maximum genetic distances of inter-species of samples (0.055). Therefore, it would be easier to distinguish Campsis Flos from the other samples using the ITS2 sequence.

Prediction and analysis of secondary structure of ITS2

The secondary structure of ITS2 provided the most accurate phylogenetic analysis (Keller et al., 2010), and its information correlated with the biological species concept (Müller et al. 2007). Studies on the secondary structures of nrRNAs have been considered important for inferring phylogenetic relationships at some taxonomic levels. The authors predicted the secondary structure of all samples using the ITS2 web site (http://its2.bioapps.biozentrum.uni-wuerzburg.de/).

Generally, secondary structures of samples contain a central loop and four helical regions (arms): I, II, III, and IV (Figure 1). There were no significant differences in the number, size and angle of the arm loops within the same species. However, there were significant differences among different species. The six species, *C. grandiflora*, *C. radicans, P. tomentosa*, *H. syriacus*, *H. syriacus L. f. var. syriacus f. amplissimus*, and *R. molle*, could be directly identified based on the nrRNA secondary structure of their ITS2 regions.

Phylogenetic analysis

The phylogenetic tree was constructed using MEGA6.0 with 1000 bootstrap replicates for the ITS2 sequences, as shown in Figure 1. The 12 samples can be clustered into classes I, II, III and IV (Figure 2). Class I included *C. radicans* and *C. grandiflora* from Genbank, Garden Hotel Suzhou, and Enshi. The samples of Campsis Flos and *P. tomentosa* were clustered into class II. Classes I, II and *R. molle* were clustered into Class III. Class III and *H. syriacus* and *H. syriacus* L. f. var. *syriacus* f. *amplissimus* were clustered into class IV. The phylogenetic tree and conventional taxonomy of the plants were consistent.

DISCUSSION

Campsis Flos has been used to encourage blood circulation and mediate blood stasis to treat diseases caused by blood stagnation. It is effective in promoting blood circulation. The flowers of *P. tomentosa, H. syriacus*, and *R. molle* are its common adulterants. *P. tomentosa* flowers can treat respiratory infections. *H. syriacus* and *H. syriacus* L. f. var. *syriacus* f. a*mplissimus* flowers can alleviate fever and promote diuresis. Rhododendri Mollis Flos are dried *R. molle* flowers, which can expel wind and remove dampness. The effects of these four common adulterants and Campsis Flos are vastly different and will produce serious consequences if misused. Although, microscopic identification can identify them, this traditional method is time-consuming and takes intensive training and experience to fully grasp (He et al.,

Table 4. Sequence alignment of *C. grandiflora* from Enshi in Hubei province and from Genbank.

Samples	Variable sites																
	17	20	21	25	27	35	44	50	67	96	168	170	194	196	235	237	241
Campsis grandiflora (Enshi)	C	G	A	G	C	T	C	C	T	C	G	A	G	A	T	C	T
Campsis grandiflora (GenBank)	T	C	C	A	G	G	G	T	C	G	A	G	C	G	C	T	C

Table 5. Kimura-2-parameter (K2P) genetic distance of Campsis Flos and its adulterants.

	Sample 3	Sample 1	Sample 2	Sample 7	Sample 8	Sample 10	Sample 9	Sample 4	Sample 5	Sample 6
Sample 3										
Sample 1	0.000									
Sample 2	0.055	0.055								
Sample 7	0.592	0.592	0.612							
Sample 8	0.579	0.579	0.600	0.005						
Sample 10	0.570	0.570	0.590	0.010	0.015					
Sample 9	0.570	0.570	0.590	0.010	0.015	0.000				
Sample 4	0.570	0.570	0.220	0.705	0.690	0.679	0.679			
Sample 5	0.281	0.281	0.260	0.754	0.738	0.726	0.726	0.040		
Sample6	0.485	0.485	0.448	0.694	0.680	0.682	0.682	0.518	0.579	

The genetic distances of *C. grandiflora* and *C.radicans* from Genebank are 0.000.

2007; Zhang et al., 2005). Therefore, molecular identification might be a good alternative. In recent years, DNA barcoding identification has emerged useful in species identification; in particular, ITS2 sequence is an efficient marker for authenticating traditional Chinese medicines. ITS2 can effectively distinguish Campsis Flos from its adulterants.

In addition, successful DNA extraction is a prerequisite for identifying herbal materials (Moyo et al., 2008). The majority of the plant materials used in herbal medicine is procured from markets in dried or powdered forms; thus, the qualities of the DNA extracted in the experiment were poor. The medicinal portions of herbs are the leaves, which provided better DNA quality.

DNA barcoding ITS2 sequence was obtained by PCR amplification and sequencing. The ITS2 secondary structures of all species are composed of a central loop (the main loop) and four helical regions (arms), and each has a different size and number of arm loops (Schultz and Wolf, 2009; Tippery and Les, 2008). The ITS2 secondary structures of *C. grandiflora* and *C. radicans* and *H. syriacus* and *H. syriacus L. f. var. syriacus f. amplissimus* have little differences. However, the ITS2 secondary structures of Campsis Flos, *P. tomentosa*, *R. molle* and *H. syriacus* were significantly different. Their sizes, positions, numbers and angles of helix arm loops, and center ring shapes are also different. The central loops of *P. tomentosa, R. molle* and *H. syriacus* have variable regions, and that of *P. tomentosa* is irregular. Thus, the prediction of ITS2 secondary structure can be used to distinguish Campsis Flos from its adulterants.

The phylogenetic trees of Campsis Flos and its adulterants that were constructed using the ITS2 sequence could easily distinguish the six species. Moreover, the clustering observed was consistent with traditional classifications. *C. grandiflora, C. radicans, P. tomentosa* and *R. molle* belong to sympetalae, and *H. syriacus* belongs to archichlamydeae. Thus, the genetic relationships among *C. grandiflora*, *C. radicans*, *P. tomentosa*

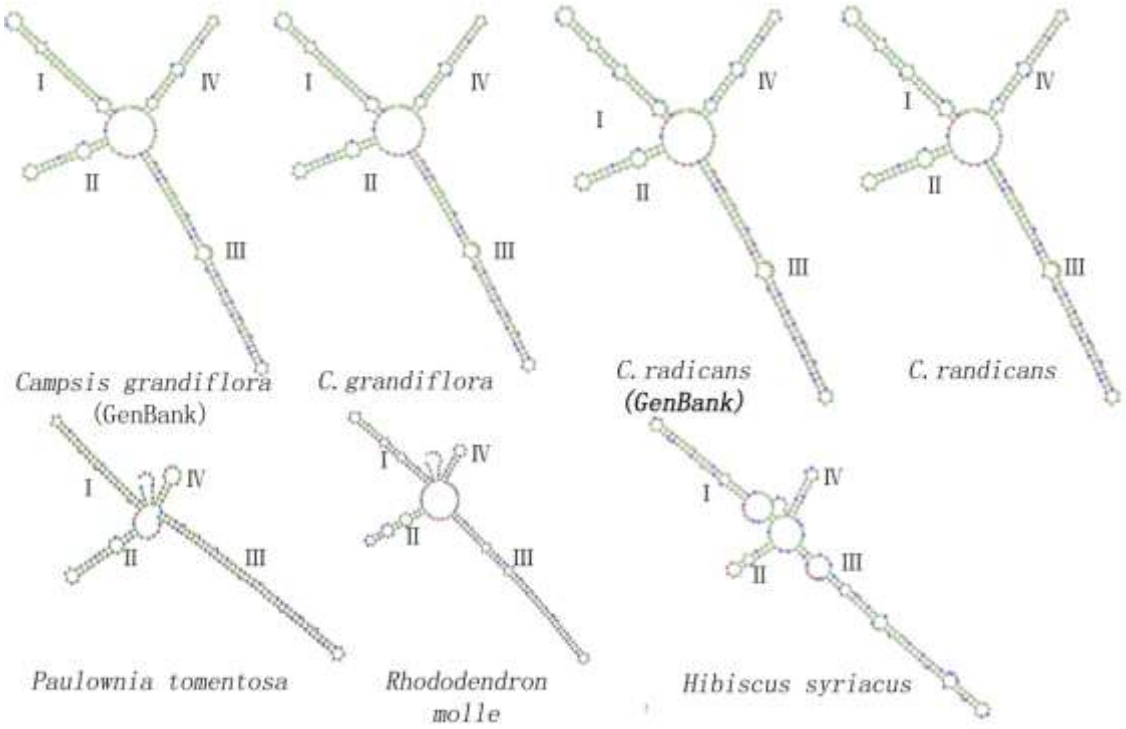

Figure 1. The ITS2 secondary structure of Campsis Flos and its adulterants. Their differences were mainly present in Helixes I, III and IV, and, therefore, were distinguishable at the molecular level.

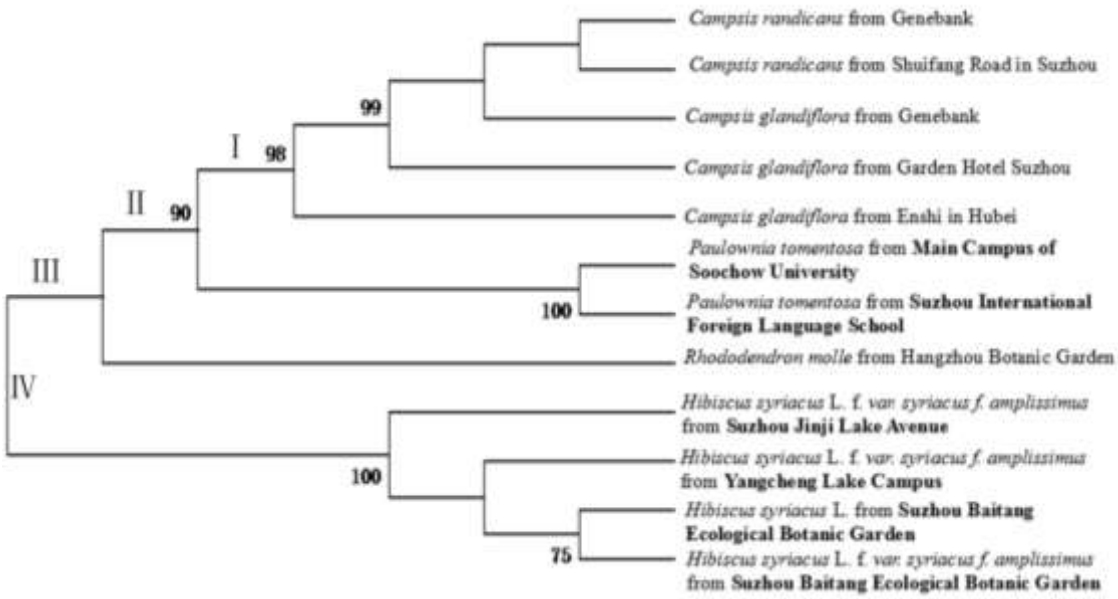

Figure 2. The NJ tree of Campsis Flos and its adulterants with the ITS2 sequences. The bootstrap scores (1000 replicates) are shown (≧75%) for each branch.

and *R. molle* were close and far from *H. syriacus*.

Adulteration is frequent in traditional Chinese medicine. If we could standardize the cultivation of Campsis Flos and establish a Good Agriculture Practice Area, the adulteration problem would be solved to a certain extent. Furthermore, ITS2 barcodes could help control the quality

of planting Campsis Flos.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

This study was funded by grants from Suzhou Science and Technology Development Project (No. Sza756531). They authors thank Professor Chunyu Liu for helping with the identification of the species of the samples.

REFERENCES

Chen SL, Yao H, Han JP, Liu C, Song JY, Shi LC, Zhu YJ, Ma XY, Gao T, Pang XH, Luo K, Li Y, Li XW, Jia XC, Lin YL, Leon C (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. Plos One 5:e8613.

Chen X, Liao B, Song J, Pang X, Han J, Chen S (2013). A fast SNP identification and analysis of intraspecific variation in the medicinal Panax species based on DNA barcoding. Gene 530:39-43.

Cui XY, Kim JH, Zhao X, Chen BQ, Lee BC, Pyo HB, Yun YP, Zhang YH (2006). Antioxidative and acute anti-inflammatory effects of *Campsis grandiflora* flower. J. Ethnopharmacol. 103:223-228.

Commission of Chinese Pharmacopoeia (2015). Chinese Phaemacopoeia, In: Part Ⅰ. The medicine science and technology press of China, Beijing. P 287.

He Y, Yang YC, Gao X (2007) Identification of Campsis Flos and its adulterants. Tianjin Pharm.19:51-53.

Keller A, Försrer F, Müller T, Dandekar T, Schultz J, Wolf M (2010). Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. Biol. Direct 5:1-12.

Keller A, Schleicher T, Schultz J, Müller T, Dandekar T, Wolf M (2009). 5.8 S-28S rRNA interaction and HMM-based ITS2 annotation. Gene 430(1):50-57.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Koetschan C, Försrer F, Keller A, Schleicher T, Ruderisch B, Schwarz R, Müller T, Wolf M, Schultz J (2010). The ITS2 database III-sequences and structures for phylogeny. Nucleic Acids Res. 38:D275-D279.

Kress WJ, Erickson DL (2007). A two-locus global DNA barcode for land plants: the coding rbcL gene.complements the non-coding trnH-psbA spacer region. PLos One 2:e508.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA 102:8369-8374.

Moyo M, Amoo SO, Bairu MW, Finnie JF, Staden JV (2008). Optimising DNA isolation for medicinal plants. S. Afr. J. Bot. 74:771-775.

Müller T, Philippi N, Dandekar T, Schultz J, Wolf M (2007). Distinguishing Species. RNA 13:1469-1472.

Pang XH, Shi LC, Song JY, Chen XC, Chen SL (2013) Use of the potential DNA barcode ITS2 to identify herbal materials. J. Nat. Med. 67:571-575.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Schultz J, Wolf M (2009). ITS2 sequence-structure analysis in phylogenetics: A how-to manual for molecular systematics. Mol. Phylogenet. Evol. 52:520-523.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA 6: molecular evolutionary genetic analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.

Tippery NP, Les DH (2008) Phylogenetics analysis of the internal transcribed spacer (ITS) region in Menyanthaceae using predicted secondary structure. Mol. Phylogenet. Evol. 49:526-537.

Yao H, Song JY, Liu C, Luo K, Han JP, Li Y, Pang XH, Xu HX, Zhu YJ, Xiao PG, Chen SL (2010). Use of ITS2 region as the universal DNA barcode for plants and animals. PLos One 5(10):e13102.

Yu HC, Wu J, Zhang HX, Zhang HS, Qiao TT, Zhang JX, Zhang GL, Sui J, Li LW, Zhang LR, Lv LX (2015). Antidepresant-like and anti-oxidative efficacy of *Campsis grandiflora* flower. J. Pharm. Pharmacol. 67:1705-1715.

Zhang GJ (2005). Microscopic identification of commonly used traditional Chinese Medicine. Chemical Industry Press, Beijing. P 234.

Zhang W,Yang S, Zhao H,Huang LQ (2016). Using the ITS2 sequence-structure as a DNA mini-barcode: A case study in authenticating the traditional medicine“FangFeng”. Biochem. Syst Ecol. 69:188-194.

Zhang ZL, Song MF, Guan YH, Li HT, Niu YF, Zhang LX, Ma XJ (2015). DNA barcoding in plants:testing the potential of proposed barcoding marker for identification of uncaria species from China. Biochem. Syst. Ecol. 60:8-14.