Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA - barcoding technique

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Submitted: 27-12-2013 Revised: 24-02-2014 Published: 26-09-2014

ABSTRACT

Background: *Peucedanum praeruptorum* L., a traditional Chinese medicine known as Qian-hu, is commonly used for dispelling wind-heat and expectorant and loss of energy. However, due to similar morphological characters and high market demand, there are many substitutes and adulterants of *P. praeruptorum*. DNA barcoding is an approach to identify species based on sequences from a short, standardized DNA region. **Objective:** To authenticate *P. praeruptorum* from its substitutes and adulterants. **Materials and Methods:** The differential identification of *P. praeruptorum* and 13 regional substitutes and 23 adulterants was investigated by means of DNA sequence analysis of internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA), a bootstrap neighbor-joining (NJ) tree according to Kimura's 2-parameter method was also calculated. **Results:** The data showed that *P. praeruptorum*, its substitutes and adulterants could be easily distinguished at the DNA level, while almost all species were well resolved, and successfully identified on the NJ tree. **Conclusion:** The ITS sequence can be used for the identification of *P. praeruptorum* and to distinguish it from common substitutes and adulterants.

Key words: Apiaceae, DNA barcoding, identification, nrDNA ITS, *Peucedanumpraeruptorum* L.

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Website: www.phcog.com

DOI: 10.4103/0973-1296.141754

Quick Response Code:

INTRODUCTION

The family Apiaceae (Umbelliferae) includes some of the world's most important medicinal and poisonous plants, such as angelica, bupleurum, anise (aniseed), celeriac, water hemlock and fool's parsley.[1] Among which, Peucedani Radix, derived from the roots of *Peucedanum praeruptorum* L., is a well-known traditional Chinese medicine called Qian-hu (Bai-hua Qian-hu), together with Peucedani decursivi Radix derived from *Angelica decursiva* L., which has been separately recorded as Zi-hua Qian-hu in the current Pharmacopoeia of the People's Republic of China.[2] *P. praeruptorum* was recorded as medicinal plant as early as the Liang Dynasty in Ming-yi-bie-lu (Apendant Records of Famous Physicians) and was also included in the ancient encyclopedia, China Compendium of Materia Medica of Ming Dynasty.[3] Phytochemical studies have shown that the active ingredients of *P.praeruptorum* include volatile oils and coumarins such as praeruptorin A and praeruptorin B,[4-6] which can deal with anemopyretic cold, cough with abundance of phlegm, impeded chest as well as removing nebula for improving eyesight. However, due to morphological similarities, high market demands and regional factors, 41 species in 16 genera of the Apiaceae are often misused or used as substitutes for *P. praeruptorum*.[3] Available criteria and methods to authenticate *P. praeruptorum*, are mainly based on its morphological characters and analysis of chemical compounds[7-10], which are susceptible to intrinsic and extrinsic factors such as the time of harvest, availability of experts and processing methods etc.[11,12] Therefore, a reliable method to discriminate *P. praeruptorum* precisely from its substitutes and adulterants is needed.

DNA barcoding, an approach to identify species based on sequences from a short, standardized DNA region, opens up a unique avenue for the identification of organisms.[13,14] Among the several candidate DNA barcodes, the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (nrDNA) has been recently

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proposed for incorporation into the core barcode, and has the potential to be used as a standard DNA barcode to identify medicinal plants and their close relatives.[15,16] Furthermore, ITS is the best and most popular marker for lower level phylogenetic analysis of Apiaceae and has demonstrated excellent reliability for species resolution.[17-21] In this study, the ITS regions of *P. praeruptorum* and its substitutes and adulterants were sequenced and compared to explore the possibility of using them to differentiate between them. The classification tree constructed by their sequences is also discussed.

MATERIALS AND METHODS

Plant material

Samples for analysis were obtained from collections of natural populations by the authors or from specimens deposited in Kunming Institute of Botany, Chinese Academy of Sciences (KUN). All accessions were identified using published keys by the first author [Table 1], and corresponding vouchers were deposited at KUN (Kunming Institute of Botany, Chinese Academy of Sciences). For *P. praeruptorum* and *A. decursiva*, seven accessions collected from different locations or downloaded from Genbank, which were examined for possible infraspecific molecular variation. In total, 50 accessions representing 38 species/variants covering *P. praeruptorum* and most of its substitutes and adulterants were included.

DNA extraction, amplification, sequencing, and data analysis

Total genomic DNA was extracted from fresh, silica-gel-dried or herbarium leaf material using the modified hexadecyltrimethylammonium bromide (CTAB) procedure of Doyle and Doyle.[22] Double-stranded DNAs of the complete ITS region (including ITS1, 5.8S and ITS-2) were polymerase chain reaction (PCR)-amplified using primers ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS-5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3').[23] These PCR reactions contained 2.0 μl of 10 × Taq DNA polymerase reaction buffer (TaKaRa Biotechnology Dalian Co., Ltd.), 2.5 mM/L of each dNTP (TaKaRa), 1.5 mM/L of $MgCl_2$, 1.0 μl of 5% dimethyl sulfoxide, 0.2 mM/L of each primer (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.), 1.5 Units of AmpliTaq DNA polymerase (TaKaRa), 1.5 μl of unquantified genomic template DNA, and sterile water to a final volume of 20 μl. The PCR parameters were as follows: Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation (94°, 45 s), annealing (55°C, 1 min) and extension (72°C, 3 min), and a final extension for 7 min at 72°C. Purifying and bidirectional sequencing were completed by Sangon Co., Ltd. DNA sequences for each accession was produced using SeqMan (DNAstar), aligned and corrected manually using the BioEdit (version 7.0.5). The Molecular Evolutionary Genetics Analysis (MEGA 4.0) was used to generate Kimura 2-parameter (K2P) distance matrices and for intraspecific and interspecific sequence similarity. Additionally, a bootstrap Neighbor-Joining (NJ) tree was calculated according to K2P method with bootstrap testing of 1000 replicates. The alignment of the ITS sequences are available upon request.

RESULTS

All samples analyzed were successfully amplified and sequenced with the universal primers "ITS4" and "ITS5". The final aligned data matrix contained 623 positions, ranging in length from 593bp to 605bp. Of these, 217 sites were parsimony informative, 318 were constant, and 88 were autapomorphic. On average, ITS1 was slightly shorter than ITS2, but it provided parsimony informative sites almost as much as ITS2. The means of guanine-cytosine (GC) content were same in ITS1 and ITS2 sequences [Table 2]. Each of the seven accessions of *P. praeruptorum*and *A. decursiva* yielded identical DNA sequences, showing that their ITS sequences are homologous regardless of geographical origin. The sequence divergence among *P. praeruptorum* and its substitutes varied from 0.00% (*P. turgeniifolium* H. Wolff) to 21.60% (*Pleurospermum bicolor* (Franch.) Norman ex Pan and Watson), while divergence values between *P. praeruptorum* and its adulterants varied from 2.50% (*P. japonicum* Thunb.) to 24.0% (*Physospermopsis delavayi* (Franch.) H. Wolff). Across the matrix, the informative and variable sites were widely dispersed, indicating that it is feasible to use sequence alignments to distinguish the *P. praeruptorum* from its substitutes and adulterants accurately. Based on the NJ tree constructed under K2P method, all accessions are resolved into eight clades with moderate to high support [Figure 1]. Except for *P. turgeniifolium*, which intermingled with accessions of *P. praeruptorum*, *P. praeruptorum* and its substitutes and adulterants could be differentiated successfully.

DISCUSSION

According to surveys, there are 11,146 medicinal plant species from 2,309 genera of 383 families in China, representing a rich biodiversity. Accurate and rapid authentication of these plants and their adulterants is difficult to achieve at the scale of international trade in medicinal plants.[15] Furthermore, many commercial products are sold either in dried form or as processed material, rendering their authentication by morphological methods very difficult, if not impossible.[24] However, DNA-based methods, e.g. barcoding can be useful in quickly and efficiently pinpointing adulterated or misidentified raw materials without further need for

Table 1: Taxa, sources, vouchers and GenBank accession numbers for species investigated in this study

Taxa	Sources/vouchers	Genbank no.
Angelica decursiva (Miq.) Franch. and Sav.	Guangxi, China, ZH7	KF806564
	Zhejiang, China, ZH8	KF806565
	*KIB, China, ZHKIB	KF806566
	Zhejiang, China, SCSB-JS0391	KF806563
	Genbank	#JN603215 #JX022912 #GU395153
c*Angelica gigas* Nakai	Genbank	#JN603218
c*Angelica megaphylla* diels	Genbank	#EU418377
c*Angelica polymorpha* Maixm.	*KIB, China, ZW01	KF806567
c*Angelica sylvestris* L.	Genbank	#HQ256681
c*Anthriscus sylvestris* subsp. *nemorosa* (Bieb.) Koso.-Pol.	Xinjiang, China, Xu256	KF806584
c*Anthriscus sylvestris* (L.) Hoffm.	Sichuan, China, ZJ0566	EU236159
c*Carum buriaticum* Turcz.	Qinghai, China, LJQ-QLS-2008-0135	KF806586
c*Conioselinum vaginatum* (Spreng.) Thell.	Xinjiang, China, ZJ0731	FJ385041
c*Cyclorhiza peucedanifolia* (Franch.) Constance	Yunnan, China, J034	FJ385042
c*Ferula olivacea* (Diels) H. Wolff	Yunnan, China, J096	FJ385043
c*Heracleum tiliifolium* H. Wolff	Genbank	#FJ812139
b*Ligusticum brachylobum* Franch.	Yunnan, China, ZJ0533	KF806583
b*Ligusticum likiangense* (H. Wolff) Pu and Watson	Yunnan, China, W810852	KF806582
b*Ligusticum daucoides* (Franch.) Franch.	Sichuan, China, ZJ0556	EU236173
c*Ligusticum pteridophyllum* Franch.	Sichuan, China, Z008	KF806581
c*Ligusticum tenuissimum* (Nakai) Kitagawa	Genbank	#JN853781
b*Ostericum citriodorum* (Hance) Yuan and Shan	Jiangxi, China, KUN4807	KF806560
c*Ostericum grosseserratum* (Maxim.) Kitagawa	Anhui, China, SCSB-JSC48	KF806562
c*Ostericum sieboldii* (Miq.) Nakai	Jiangsu, China, KUN3480	KF806561
b*Peucedanum dissolutum* (Diels) H. Wolff	Genbank	#EU418388
b*Peucedanum formosanum* Hayata	Guangxi, China, LZ0903	KF806571
c*Peucedanum japonicum* Thunb.	Zhejiang, China, LZ0916	KF806570
c*Peucedanum longshengense* Shan and Sheh	Guangxi, China, LZ0912	KF806572
b*Peucedanum medicum* Dunn	Hubei, China, KUN3215	KF806573
b*Peucedanum medicum* var. *gracile* Dunn ex Shan and Sheh	Genbank	#JF977816
Peucedanum praeruptorum Dunn	Jiangxi, China, BH1	KF806579
	Zhejiang, China, BH2	KF806580
	Shanxi, China, B510	KF806577
	Shanxi, China, B511	KF806578
	Genbank	#EU418383 #EU592009 #DQ132871
b*Peucedanum rubricaule* Shan and Sheh	Yunnan, China, Yangqe1843	KF806574
c*Peucedanum terebinthaceum* (Fisch. ex Trevir.) Ledeb.	Jilin, China, LZ0922	KF806575
c*Peucedanum terebinthaceum* var. *deltoideum* (Makino ex Y. Yabe) Makino	Liaoning, China, KUN1584	KF806576
b*Peucedanum turgeniifolium* H. Wolff	Sichuan, China, ZJ0634	EU236187
b*Peucedanum wawrae* (H. Wolff) Su ex Sheh	Anhui, China, LZ0917	KF806568
b*Peucedanum wulongense* Shan and Sheh	Chongqing, China, LZ20090935	KF806569
c*Physospermopsis delavayi* (Franch.) H. Wolff	Yunnan, China, J033	FJ385056
c*Pimpinella diversifolia* DC.	Yunnan, China, ZJ0518	KF806585
b*Pleurospermum bicolor* (Franch.) Norman ex Pan and Watson	Yunnnan, China, KUN23302	KF806587
c*Selinum cryptotaenium* H. Boissieu	Yunnan, China, ZJ810856	EU236206
c*Meeboldia yunnanensis* (H. Wolff) Constance and Pu	Yunnan, China, ZJ0673	EU236178

#Accession downloaded from GenBank; *Samples cultivated in Kunming Institute of Botany; bsubstitutes; c adulterants

time- and resource-consuming morphological, physical, and phytochemical examinations.[25,26]

A suitable barcode must exhibit high interspecific but low intraspecific divergence.[27] ITS was initially proposed as a universal DNA barcode for plants because of its high sequence divergence[28], and it also has been successfully used as a genetic marker for molecular authentication and identification of several medicinal plants. *Panax ginseng* C.A.Mey.[29,30], *Dendrobium* Species[25,26,31], *Euphorbia pekinensis*[32],

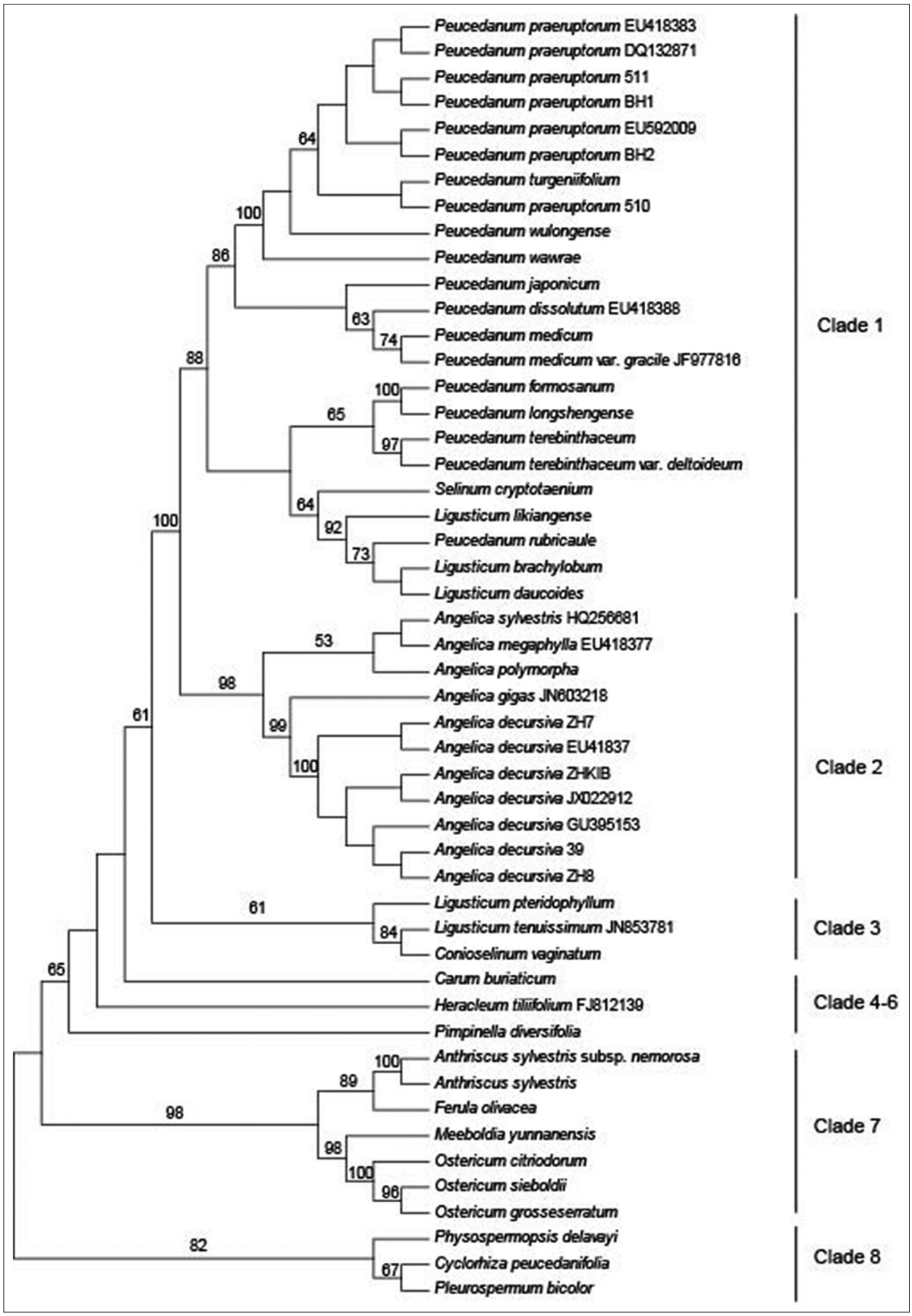

Figure 1: Classification tree of ITS sequences using the NJ method. Branch length was calculated by Kimura's 2-parameters method. Bootstrap (1000 replicates) analysis was performed to estimate the confidence of the topology of the consensus tree

Bupleurum species[33], *Chimaphila* species[34] and *Gentianopsis paludosa* (Hook. f.) Ma[24] are all successful examples. Furthermore, Chen *et al.* tested the discrimination ability of ITS2 (4800 species from 753 distinct genera) and found that it has the potential to be used as a standard DNA barcode to identify medicinal plants and their closely related species.[15] Li *et al.* proposed that ITS/ITS2 should be incorporated into the core barcode for seed plants after investigating several candidate barcodes.[16] In our barcoding results, a single-region of ITS sequence can distinguish *P. praeruptorum* from its substitutes and adulterants. This was supported by sequence alignment analyses, which revealed the high sequence variation to be sufficient for species identification.

Traditionally, because of their similar effects on dispelling wind-heat, expectorant and loss of energy, *A. decursiva*, together with *P. praeruptorum* were both regarded as certified products of Qian-hu.[35] However, subsequent chemical analysis revealed that they differ greatly in type of coumarins. Bai-hua qian-hu mainly contains angle-type dihydro-pyran-coumarins, while line-type furan- or pyran- coumarins are the main components of Zi-hua qian-hu[4,5], and they have been separately recorded in the current Pharmacopoeia of the People's Republic of China.[2] *Angelica decursiva* was previously ascribed to the genus *Peucedanum* as *P. decursivum* (Miq.) Maxim. Recent phylogenetic studies considered that *A. decursiva*/*P. decursivum* should be classified into *Angelica*.[36] In our results, seven accessions of *A. decursiva* formed a well-supported clade distant from *P. praeruptorum*, with the divergence value of 6.20%. In the background of Chinese herb standardization, it is difficult to specify a unanimous quality standard for Qian-hu from two different resources. Therefore, we consider that it is more reasonable to consider *A. decursiva* to be a regional substitute for *P. praeruptorum*.

Through historical textual research and chemical analyses, thirteen species, *Ligusticum brachylobum* Franch., *L. likiangense* (H. Wolff) Pu and Watson, *L. daucoides* (Franch.) Franch., *Ostericum citriodorum* (Hance) Yuan and Shan, *Peucedanum dissolutum* (Diels) H. Wolff, *P. formosanum* Hayata, *P. medicum* Dunn, *P. medicum* var. *gracile* Dunn ex Shan and Sheh, *P. rubricaule* Shan and Sheh, *P. turgeniifolium* H. Wolff, *P. wawrae* (H. Wolff) Su ex Sheh, *P. wulongense* Shan and Sheh and *Pleurospermum bicolor* are regarded as regional substitutes for *P. praeruptorum*.[3] Sequence divergence values between *P. praeruptorum* and these substitutes, all of which mainly clustered into Clade 1 except for *Pleurospermum bicolor* and *Ostericum citriodorum* [Figure 1], ranged from 0.00% to 21.60%. *Pleurospermum bicolor* is a traditional medical plant used by the Naxi ethnic group as Qian-hu. Despite their great pair-wise distance (21.60%), it can be substituted for *P. praeruptorum* because of similar active ingredients.[37] Except for *P. turgeniifolium*, all the other substitutes were well resolved on the NJ tree. *Peucedanum turgeniifolium* occurs in S Gansu (Jone, Têwo) and northern Sichuan and is important in Sichuan folk medicine. According to Rao *et al.*, it has chemical constituents similar to those of *P. praeruptorum* and can be regarded as a substitute.[38] In our study, the sequence divergence between them was 0.00% (the divergence values between them for other barcodes such as *psbA-trnH*, *matK* and *rbcL* were also zero, unpublished data). As *P. praeruptorum* is distributed widely in China, covering Gansu and Sichuan, we consider *P. turgeniifolium* to be a geographical variety of *P. praeruptorum* pending further investigation.

Table 2: Sequences characteristics of ITS in this study

Sequences characteristics	ITS1	5.8S	ITS2
Length range (bp)	213-221	162-163	217-225
Constant sites No. (%)	96 (41.92)	144 (88.34)	78 (33.77)
Parsimony-informative sites No. (%)	102 (44.54)	11 (6.75)	104 (45.02)
Autapomorphic sites No. (%)	31 (13.53)	8 (4.90)	49 (21.21)
G+C content mean (%)	55.60	53.90	55.60
Sequence divergence range (%)	0.00-37.80	0.00-5.20	0.00-38.70

Pairwise sequence divergence estimates ranged from 2.50% to 24.0% of nucleotides within *P. praeruptorum* and its adulterants, the latter of which were scattered throughout the NJ tree (Clade 1–Clade 8) [Figure 1]. All adulterants were well resolved and could be distinguished from *P. praeruptorum*. Most of the adulterants, such as *P. terebinthaceum* (Fisch. ex Trevir.) Ledeb., *Ligusticum pteridophyllum* Franch. and *Angelica sylvestris* (L.) Hoffm., also used differed medicinally from *P. praeruptorum*, and so should be used under their original herbal medicine names.

In conclusion, *Peucedanum praeruptorum* could be identified by analysis of a single DNA barcoding–ITS sequence that provides enough variability for authentication in contrast to the difficulties in using morphological characters. We consider nrDNA ITS sequence analysis to be reliable and convenient for authenticating *P. praeruptorum* and its substitutes, adulterants and other medicinal species.

ACKNOWLEDGMENTS

The authors thank David E. Boufford from Harvard University Herbaria for language polishing. This work was supported by the National Natural Science Foundation of China (no. 31000098), the Open-access Project of the State Key Laboratory of Systematic and Evolutionary Botany (LSEB) and Open Research Foundation of Yunnan Key Laboratory of Pharmacology for Natural Products (No. 2013G006).

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Cite this article as: Zhou J, Wang W, Liu M, Liu Z. Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum* and its substitutes and adulterants by DNA - barcoding technique. Phcog Mag 2014;10:385-90.

Source of Support: Nill, **Conflict of Interest:** None declared.