Molecular identification of Achyranthis Bidentatae Radix by using DNA barcoding

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Genet. Mol. Res. 15 (2): gmr.15028783
Received May 16, 2016
Accepted June 3, 2016
Published June 24, 2016
DOI http://dx.doi.org/10.4238/gmr.15028783

ABSTRACT. Achyranthis Bidentatae Radix has a long history in China as a commonly used herb that can be used to treat various diseases, including those related to the liver, muscles, bones, and kidneys. Recently, an increase in the number of adulterants has been reported, which affects the clinical safety of Achyranthis Bidentatae Radix. To identify adulterants of Achyranthis Bidentatae Radix, we collected samples from major regions and conducted an in-depth genetic comparison of the herb and its commonly used adulterants. We amplified and sequenced three genomic regions, internal transcribed spacer (ITS), *psb*A-*trn*H, and internal transcribed spacer 2 (ITS2), to confirm whether ITS2 is a suitable identifier for Achyranthis Bidentatae Radix. Results showed that the ITS2 sequence length of Achyranthis Bidentatae Radix was 199 bp, with no variation between samples. The

inter-specific genetic distance of ITS2 between Achyranthis Bidentatae Radix and its adulterants was 0.390. Neighbor-joining trees showed that Achyranthis Bidentatae Radix and its adulterants are easily differentiated by monophyly. In conclusion, ITS2 regions accurately and effectively distinguished between Achyranthis Bidentatae Radix and its adulterants.

Key words: Achyranthis Bidentatae Radix; DNA barcoding; ITS2; Species identification

INTRODUCTION

Achyranthis Bidentatae Radix is the dried root of *Achyranthes bidentata* Blume (Amaranthaceae) that is used in traditional Chinese medicine (TCM). The herb benefits the liver, kidneys, muscles, and bones, and can also treat other conditions such as backache, gonyalgia, edema, headache, vertigo, and toothache (Chinese Pharmacopoeia Commission, 2015). In recent years, the study of Achyranthis Bidentatae Radix has incorporated medical theories, chemical components, and other aspects. Studies indicate that this medicinal plant contains steroids, saponins, polysaccharides, flavones, and other chemical constituents (Meng and Li, 2001).

Achyranthis Bidentatae Radix is a commonly used herb and is one of the widely known "Four Huái Medicines". Listed as a rare Chinese herb, it needs to be developed mostly by the State Administration of TCM. Currently, most commercial Achyranthis Bidentatae Radix is artificial; cultivated products from Henan are regarded as the highest quality (Chen, 2011). However, the rapid increase in the consumption of this herb has led to more adulterants being introduced for medicinal purposes, such as the roots of *Arctium lappa*, *A. tomentosum*, and *A. minus*. These are often confused with Achyranthis Bidentatae Radix. Unauthentic *Cyathula prostrata* and *Achyranthes aspera*, which are used as folk herbal medicine, are also common adulterants. In the Chinese Pharmacopoeia Commission (2015), Cyathulae Radix has a similar name to Achyranthis Bidentatae Radix; however, they have different functions. Thus, these two herbs and their clinical uses must be identified and distinguished in herbal markets (Liao et al., 2013). Achyranthis Bidentatae Radix has become difficult to identify, seriously affecting the clinical use and safety of this herb in TCM. The rapid and accurate identification of this herb, and its adulterants, is key to controlling its quality and safety. At present, identification methods rely mainly on the characterization of properties, microstructural methods (He et al., 2011; Guo, 2013), and fingerprint identification (Wang et al., 2003; Guo et al., 2010; Zhao et al., 2012). However, these techniques only ensure the clinical safety of the herb to a certain degree.

The rapid development of molecular techniques in recent years has enabled the widespread use of discrimination techniques, such as restriction fragment length polymorphism analysis (Wiriyakarun et al., 2013), random amplified polymorphic DNA (Zheng et al., 2015), and application of simple sequence repeats (Xie et al., 2010). These approaches do not have the same weaknesses as property characterization and can complement traditional discrimination methods. However, the repeatability and universal property of these techniques renders them inefficient. They also lack international application platforms so that promoting their wider use is impractical.

A relatively new molecular diagnostic technique for identifying species is DNA barcoding, which uses a short length of standardized genetic marker (Hebert, et al., 2003a; Chen,

2012; Fišer Pečnikar and Buzan, 2014). DNA barcoding is not influenced by external environments, development phases, or morphological characteristics, and is therefore preferable for standardized species identification (Gregory, 2005). From its inception, DNA barcoding has drawn attention. This study used the DNA fragments (Hebert et al., 2003a,b) internal transcribed sequence (ITS), *mat*K, *rbc*L, and *psb*A-*trn*H, as candidate plant barcodes. However, no authentic or universal system of plant barcoding has been agreed (Pennisi, 2007; Lahaye et al., 2008; Liu et al., 2012; Sun et al., 2013; Christina and Annamalai, 2014). Yao proposed ITS2 as a universal barcode to identify botanical species (Yao et al., 2010). The Chinese Plant BOL Group suggested that ITS/ITS2 serves as core barcodes for seed plants (Li et al., 2011). Chen et al. (2010, 2013) first presented a medicinal plant DNA barcoding identification system, with ITS2 as the core and *psb*A-*trn*H as the supplement, using a large number of samples and comprehensive screening of DNA barcodes. The potential for ITS2 to identify species has recently been confirmed, and this DNA fragment has been applied to various medicinal plants and materials (Han et al., 2013; Hou et al., 2013a,b, 2014a,b; Richardson et al., 2015; Santos et al., 2015). Based on these studies, we collected samples of Achyranthis Bidentatae Radix and its adulterants to rapidly and correctly distinguish this medicinal plant.

MATERIAL AND METHODS

Materials

A total of 55 samples, including experimental samples and sequences downloaded from GenBank, were used. Samples were comprised of 22 *A. bidentata*, 10 *Arctium lappa*, 2 *A. tomentosum*, 3 *A. minus*, 16 *Cyathula officinalis*, 1 *C. prostrata*, and 1 *Iresine angustifolia*. All samples were identified by Prof. LIN Yu-Lin from the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Science. Voucher specimens were deposited in the IMPLAD herbarium in Beijing and the Agricultural College of Henan University of Science and Technology. Materials and GenBank numbers of sequences are shown in Table 1.

Methods

DNA extraction

Surfaces of Achyranthis Bidentatae Radix herbs were cleaned. Approximately 40 mg of the medicinal materials and PVP-40 (i.e., 10% of samples) were mixed together in a 1.5-mL centrifuge tube. Total genomic DNA was extracted using a plant genomic DNA kit (Tiangen Biotech Co., Ltd., China) after grinding for 2 min at 30 oscillations/s using a DNA extraction grinder (MM 400; Retsch, Germany). Extracts were heated in a water bath at 56°C 12 h, and the subsequent steps performed following the manufacturer instructions.

PCR amplification and sequencing

Amplification systems, primer sequences, and amplification programs for PCR amplification of the three regions (ITS, ITS2, *psb*A-*trn*H) followed Chen et al. (2010). Purified PCR products were directly sequenced bi-directionally with an ABI 3730xl DNA Analyzer (Applied Biosystems Co., USA).

Table 1. Samples and GenBank No. of ITS2 used in this study.

Latin name	Voucher No.	GenBank No.	Locality
Achyranthes bidentata	YC0097MT01	KC898552	Luoyang, Henan
A. bidentata	YC0097MT02	KC898553	Luoyang, Henan
A. bidentata	YC0097MT03	KC898554	Luoyang, Henan
A. bidentata	YC0097MT04	KC898555	Wenxian, Henan
A. bidentata	YC0097MT05	KC898556	Weixian, Henan
A. bidentata	YC0097MT06	KC898557	Qinyang, Henan
A. bidentata	YC0097MT07	KC898558	Qinyang, Henan
A. bidentata	YC0097MT08	KC898559	Wuzhi, Henan
A. bidentata	YC0097MT09	KC898561	Jiaozuo, Henan
A. bidentata	YC0097MT10	KC898560	Bozhou, Anhui
A. bidentata	YC0097MT11	KC898562	Bozhou, Anhui
A. bidentata	YC0097MT12	KC898563	Bozhou, Anhui
A. bidentata	YC0097MT13	KC898564	Bozhou, Anhui
A. bidentata	YC0097MT14	KC898565	Beijing
A. bidentata	YC0097MT15	KC898566	Beijing
A. bidentata	YC0097MT16	KC898567	Beijing
A. bidentata	YC0097MT19	KC898568	Anguo, Hebei
A. bidentata	YC0097MT20	KC898569	Anguo, Hebei
A. bidentata	YC0097MT21	KC898570	Anguo, Hebei
A. bidentata	YC0097MT22	KC898571	Beijing
A. bidentata	YC0097MT23	KC898572	Beijing
A. bidentata	YC0097MT24	KC898573	Anguo, Hebei
Arctium lappa	PS0668MT05	GU724309	Nanning, Guangxi
A. lappa	PS0668MT02	GU724308	Nanning, Guangxi
A. lappa	PS0668MT09	GQ434509	Beijing
A. lappa	PS0668MT10	GQ434510	Chongqing
A. lappa	-	FJ449883	GenBank
A. lappa	-	FJ449872	GenBank
A. lappa	-	FJ449890	GenBank
A. lappa	-	FJ449886	GenBank
A. lappa	-	FJ449866	GenBank
A. lappa	-	FJ449868	GenBank
Cyathula offinalis	YC0022MT01	KC898649	Luoyang, Henan
C. offinalis	YC0022MT02	KC898642	Luoyang, Henan
C. offinalis	YC0022MT03	KC898643	Luoyang, Henan
C. offinalis	YC0022MT04	KC898644	Luoyang, Henan
C. offinalis	YC0022MT05	KC898645	Qinyang, Henan
C. offinalis	YC0022MT06	KC898646	Qinyang, Henan
C. offinalis	YC0022MT07	KC898647	Anguo, Hebei
C. offinalis	YC0022MT08	KC898650	Anguo, Hebei
C. offinalis	YC0022MT09	KC898648	Anguo, Hebei
C. offinalis	YC0022MT10	KC898636	Bozhou, Anhui
C. offinalis	YC0022MT11	KC898637	Bozhou, Anhui
C. offinalis	YC0022MT12	KC898638	Beijing
C. offinalis	YC0022MT13	KC898639	Beijing
C. offinalis	YC0022MT14	KC898640	Beijing
C. offinalis	YC0022MT15	KC898641	Beijing
C. offinalis	-	DQ497187	GenBank
C. prostrata	-	AY174421	GenBank
Iresine angustifolia	-	AY255508	GenBank
Arctium tomentosum	-	GQ281034	GenBank
A. tomentosum	-	GQ281035	GenBank
A. minus	-	HM921426	GenBank
A. minus	-	KC603906	GenBank
A. minus	-	AF319103	GenBank

Data analysis

After sequencing, forward and reverse trace files were trimmed and assembled using the CodonCode Aligner v3.7.1 (CodonCode Co., USA). The ITS2 region was annotated with HMMER software using the ITS2 database. The ITS and *psb*A-*trn*H regions were annotated using GenBank. Candidate regions were preliminarily evaluated according to the

efficiency of PCR amplification and the success rate of sequencing. The candidate region was used for further analysis. Genetic distances were calculated using molecular evolutionary genetics analysis (MEGA5.0) (Tamura et al., 2011), according to the Kimura 2-parameter (K2P) model. Neighbor-Joining (NJ) trees were constructed with 1000 bootstrap replicates. In addition, BLAST was used to identify Achyranthis Bidentatae Radix and its adulterants using the species identification system for TCM (http://www.tembarcode.cn/) (Chen, 2012). BLAST comparison and NJ trees were used to distinguish Achyranthis Bidentatae Radix and its adulterants.

RESULTS

DNA extraction and PCR amplification

DNA electrophoresis revealed that some stripes were diffuse, which showed the medicinal materials DNA barcodes; those that had been deposited for an extended time had partly degraded. The OD_{260}/OD_{280} ratio of most of the extracted genomic DNA, measured with NanoDrop, ranged from 1.6-1.8, which explained the high-quality bi-directional trace files obtained from the ITS regions. Results showed the presence of a few mixed carbohydrates with a specific OD_{260}/OD_{280} ratio of genomic DNA from only five samples over 2.0. Genomic DNA concentrations in all the samples exceeded 10 ng/μL. In addition, the outcomes of PCR amplification indicated that ITS2 was amplified successfully, however, some degraded genomic DNA, or carbohydrates, and other secondary metabolites were also present.

Efficiency rates of amplification and sequencing of ITS, ITS2, and *psb*A-*trn*H are shown in Figure 1. Results indicated that the amplification and sequencing success rates of ITS2 were the highest (100%), whereas the success rates for ITS were 50 and 41%, respectively. Amplification and sequencing success rates for *psb*A-*trn*H were 59 and 55%, respectively. Given the low PCR amplification and sequencing success rates for ITS and *psb*A-*trn*H, we could not consider them as suitable DNA barcodes to identify Achyranthis Bidentatae Radix and its adulterants. Thus, we performed an in-depth analysis of ITS2 only.

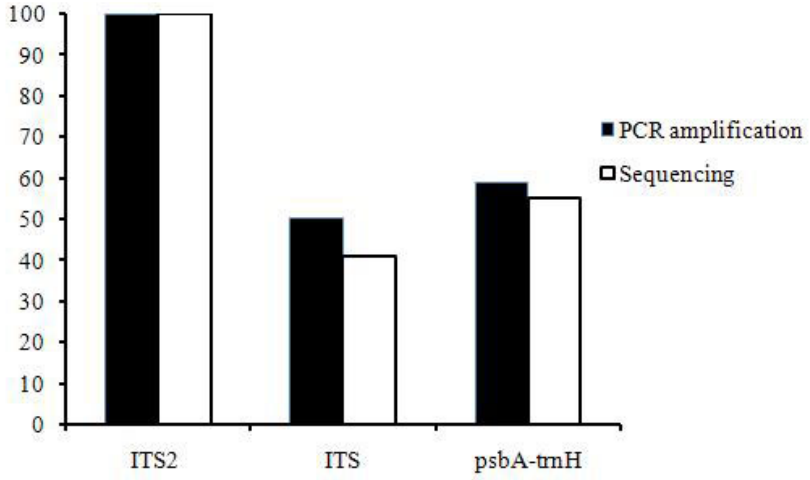

Figure 1. Efficiency of amplification and sequencing for each candidate region.

Analysis of intra-specific variation of Achyranthis Bidentatae Radix

A total of 22 medicinal materials were collected from different sources (Table 1). All ITS2 sequence lengths were 199 bp, the average GC content was 57.8%, and there was no intra-specific variation. Cyathulae Radix, another medicinal material in the pharmacopoeia, is an herb that is often confused with Achyranthis Bidentatae Radix. A total of 16 Cyathulae Radix were chosen from various sources. Their ITS2 sequence lengths were 201 bp, and the average GC content was 54.2% (Table 2). Only two variation sites (G-A variation at the 15th site) were found in the ITS2 regions: A in the 151st sites of sequences KC898641 and KC898642. However, the corresponding sites of the other 14 samples were G.

Table 2. Sequence characteristics of ITS2 in *Achyranthes bidentata* and its related species.

Sequence characteristics	ITS2
Length in *A. bidentata* (bp)	199
Length in *C. offinalis* (bp)	201
Length in all taxa (bp)	199-221
G+C content range in *A. bidentata* (%)	57.8
G+C content range in *C. offinalis* (%)	54.2
G+C content range in all taxa (%)	53.7-64.1 (58.2)

Analysis of inter-specific variation of Achyranthis Bidentatae Radix and its adulterants

From the K2P model, the results of genetic distance analysis are shown in Table 3. No intra-specific variations were observed in the ITS2 regions of the 22 medicinal materials, and the K2P genetic distance was 0. For the ITS2 sequences of 16 Cyathulae Radix, the genetic distances ranged from 0 to 0.005, with an average of 0.001. The length of the ITS2 region of Achyranthis Bidentatae Radix and its adulterants, 55 in total, ranged from 199 to 221 bp. The GC content ranged from 53.7 to 64.1, with an average of 58.2. Average inter-specific K2P distance was 0.390, and the minimum inter-specific distance was 0.011, which was markedly larger than the largest intra-specific distance between Achyranthis Bidentatae Radix and Cyathulae Radix (Tables 2 and 3).

Table 3. Intraspecific and interspecific genetic distances between *Achyranthes bidentata* and its adulterants.

K2P genetic distances	Range of genetic distances (mean)
Intraspecific distances of *A. bidentata*	0
Intraspecific distances of *C. offinalis*	0-0.005 (0.001)
Interspecific distances between *A. bidentata* and its adulterants	0.011-0.646 (0.390)

Identification of Achyranthis Bidentatae Radix and its adulterants

There were 55 ITS2 sequences belonging to Achyranthis Bidentatae Radix, Cyathulae Radix, and its common adulterants. Phylogenetic trees were constructed using the NJ method based on the K2P model (Figure 2). Results indicated that Achyranthis Bidentatae Radix formed one clade that could be successfully distinguished from its adulterants using the ITS2 regions with a success rate of 100%. *C. officinalis* and *C. prostrata* formed one clade, which could also be distinctly identified. *A. lappa*, *A. tomentosum*, and *A. minus* also formed one

monophyletic clade. In conclusion, NJ trees were able to distinguish Achyranthis Bidentatae Radix and its adulterants. Moreover, the results indicated that the ITS2 regions had the highest identification efficiency (100%) when the BLAST method was applied, using the species identification system for TCM (http://www.tcmbarcode.cn/). Thus, Achyranthis Bidentatae Radix could be distinguished unambiguously from its adulterants using the ITS2 sequence.

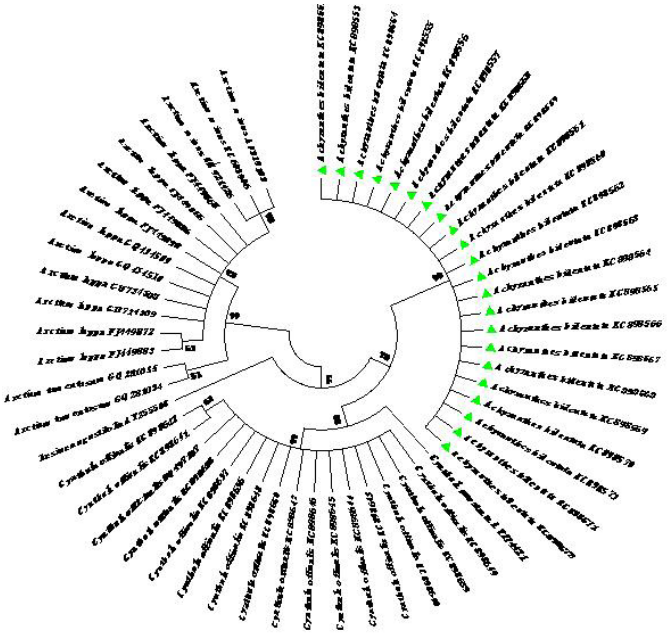

Figure 2. Phylogenetic tree of *Achyranthes bidentata* and its adulterants constructed with the ITS2 sequences using NJ method. Bootstrap scores (1000 replicates) for each branch are shown (≥50%).

DISCUSSION

DNA extraction

The basis for studying, and successfully applying, DNA barcoding techniques in medicinal materials depends on acquiring genomic DNA (Hou et al., 2013b). Genomic DNA of Chinese medicinal materials can be easily extracted from flowers and leaves, and the method is well developed (Chen, 2012) [13]. However, for dried roots and stems, the technique is more difficult as methods used to isolate DNA in secondary metabolites, and different roots and stems vary. In this study using common kit methods, we added 10% PVP-40 when extracting DNA to account for the high polysaccharide and polyphenol content in Achyranthis Bidentatae Radix. By contrast, 10% PVP-40 can be used to combine phenolic compounds into unstable complexes to remove polyphenols and eliminate polysaccharides. Thus, it can reduce the interference of polysaccharides and polyphenols and increase the purity of DNA. Similar studies on medicinal materials in *Gentiana macrophylla* have been reported (Luo et al., 2012). In addition, heating DNA at 56°C overnight also increased the efficiency of isolating DNA.

Selection and determination of identifying barcoding regions

The assessment of candidate regions in the study of plant DNA barcodes has gained worldwide interest. Many fragments, including ITS, ITS2, *mat*K, *rbc*L, and *psb*A-*trn*H, are regarded as candidate sequences to identify plants (Liu et al., 2012; Sun et al., 2013; Christina and Annamalai, 2014). This study compared three candidate regions (ITS, ITS2, and *psb*A-*trn*H). ITS and *psb*A-*trn*H exhibited inefficient amplification and sequencing, which may be attributed to the length of ITS. Extracting DNA from dried Achyranthis Bidentatae Radix resulted in low efficiency and severe degradation. The *psb*A-*trn*H content in the roots and stems of Achyranthis Bidentatae Radix, representing a genomic sequence from the chloroplast, was particularly low, and was mixed with various secondary metabolites, which may explain the inefficiency of PCR amplification of *psb*A-*trn*H. By contrast, ITS2, a multi-copy sequence in karyogenes, was short in length. Although genomic DNA was partly degraded, we easily achieved 100% amplification and sequencing success rates for ITS2 regions. We propose ITS2 as a distinct barcode for Achyranthis Bidentatae Radix to analyze amplification and sequencing. Our results indicated that the ITS2 region can accurately and efficiently discriminate Achyranthis Bidentatae Radix and its adulterants.

Comparison of authentication methods for Achyranthis Bidentatae Radix

Achyranthis Bidentatae Radix is a common and extensively used TCM in clinical settings. Recently, numerous adulterants have been commercially available, seriously affecting the clinical safety of Achyranthis Bidentatae Radix. At present, the identification of Achyranthis Bidentatae Radix and its adulterants is mainly conducted through the characterization of its properties, microstructural methods, and fingerprint identification (He et al., 2011; Zhao et al., 2012). Both methods depend on the skills of TCM clinicians. In addition, identification is usually affected by subjective factors. Fingerprint identification is also difficult as it is complex. The recent rapid development of molecular techniques to differentiate species with high accuracy and utility avoids the limitations of the aforementioned methods and provides molecular evidence for the accurate identification of medicinal plants. This study is the first to apply DNA barcoding to identify Achyranthis Bidentatae Radix and its adulterants. Results showed that the ITS2 sequence successfully discriminated this herb and its adulterants and may be an effective supplement to traditional methods. It also provides the foundation for the molecular identification of Chinese medicine and Chinese patent drugs.

ACKNOWLEDGMENTS

The authors thank J.Y. Song, H. Yao. and Y.L. Lin (Chinese Academy of Medical Sciences, Beijing, China) for useful comments and morphological confirmation of medicinal materials. Research supported by the National Natural Science Foundation of China (#U1404829) and the Key Projects in Henan Province Department of Education (#12B180007, #14A180006).

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