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Species identification of poisonous medicinal plant using DNA barcoding

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[ABSTRACT] The aim is to select a universal DNA barcode for identifying all poisonous medicinal plants in Chinese pharmacopoeia and their poisonous related species or adulterants. We chose 4 commonly used regions as candidate DNA barcodes (*ITS2*, *psbA-trnH*, *matK* and *rbcL*) and compared their identification efficiency in 106 species from 27 families and 65 genera totally. Data analysis was performed including the information of sequence alignment, inter/intra-specific genetic distance and data distribution, identification efficiency and the situation of Neighbor-Joining (NJ) phylogenetic trees. We found *ITS2* sequence region had high variation, stable genetic distance and identification efficiency relatively. The topological structure of NJ phylogenetic tree showed monophyletic. Our findings show that *ITS2* can be applied as a universal barcode for identifying poisonous medicinal plants in Chinese pharmacopoeia and their poisonous related species or adulterants.

[KEY WORDS] Sequence; Poisonous herbal medicine; Genetic distance; DNA barcoding; ITS2; Blast

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Introduction

Many poisonous plants have been used as medical materials for a rather long period since ancient time such as *Croton tiglium*, *Datura metel* and *Strychnos nuxvomica* which were noted both in Traditional Chinese Medicine (TCM) and the Ayurveda of India [1]. Chinese Pharmacopoeia (2015) contained more than 75 poisonous herbal materials whose original plants cover 34 families, 54 genus and 77 species including *Pinellia ternata* (Araceae) and *Sinopodophyllum hexandrum* (Berberidaceae) *et al*.. The rhizome of *Pinellia ternata* is currently used for its anti-atherosclerosis and hypolipidemic effect [2]. Podophyllotoxin extracted from *Sinopodophyllum hexandrum* and *Podophyllum peltatum* was used in anticancer therapy for its role in cell cycle arrest and suppression of the formation of the mitotic-spindles microtubule [3].

Nowadays, concerns have been increasing on the safety of poisonous herbal materials for misidentifying, misusing or being mistaken into other herbal materials. Since most commercial herbal productions in the market are particles, slices or even powders, and especially poisonous materials are necessary to be additionally processed, it is commonly seen that it has been misused or mixed with similar relative species intentionally or accidentally, therefore species identification is quite significant especially for poisonous medicinal plants. Mistaken identification, misuse and mixture of poisonous herbal medicine or toxic substances were the main causes of poisoning accidents, such as the accident of aristolochic acids (AAs) caused by misusing *Aristolochia fangchi* as *Stephania tetrandra* leading to a number of women suffering from upper tract urothelial carcinomas (UTUC) [4]. *Brugmansia arborea* mixed into *Datura stramonium* will cause poisoned response by scopolamine [5]. *Persicae semen* and *Armeniacae semen amarum* have similar appearance and texture but completely different clinical applications, and *Armeniacae semen amarum* is even with mild toxicity. There will be certain risk if *Armeniacae semen amarum* mixed in *Persicae semen* [6]. Among poisonous herbal materials, *Euphorbia pekinensis* and

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Stellera chamaejasme are also easy to be mixed into each other [7]. Furthermore, substitution and filler also affect the quality of herbal materials in the market [8].

As for the identification of poisonous herbal materials, traditional methods are based on the differences of botanical morphology, microscopic characteristics and physicochemical properties. Morphological diagnosis has four significant limitations: phenotypic plasticity and genetic variability can lead to misidentification; morphologically cryptic taxa are common; morphological keys are often effective only in flower and fruit stage; and extremely lack of professional taxonomists. In addition, microscopic and physicochemical identification need a high level of expertise yet sample repeatability is not high [9-10]. Besides, the geographical and climatic factors also result in the compositional difference of herbal materials leading to difficulties in herbal materials identification objectively by physicochemical methods [11]. Compared with traditional methods, molecular methods have special genetic diversity, specificity and population differentiation. Basing on molecular biological techniques, Paul Hebert put forward the term of DNA barcode using recognized standard, relatively short DNA sequence fragment as molecular maker for species identification [12]. In other words, DNA barcoding is a microgenomic identification system which has the advantage of good repeatability and high universality. This method was easy to be popularized and standardized by building a unified database and identification platform. And the identification efficiency of DNA barcoding method will not be affected by experience nor environmental factors [13]. Meanwhile, specific barcodes can be used to make accurate identification at species-level and even in population level [14]. Among currently applied DNA barcodes, Consortium for the Barcode of Life (CBOL) adopted combination of large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) plus and maturase K gene (*matK*) as standard plant DNA barcode [15-16]. In fact, Ka-Lok Wong found that *rbcL* and *matK* had no significant advantages over *trnH-psbA*, *trnL-F*, *rpl36-rps8*, *ITS* and *5S rRNA* in identifying *Gentiana* species from their adulterations [17]. Consequently, CHEN *et al*. proposed internal transcribed spacer 2 (*ITS2*) sequence as the universal DNA barcode for medicinal plants [18-19]. Moreover, *psbA-trnH* spacer was suggested as the complementary barcode of *ITS2*. The identification rate of *psbA-trnH* + *ITS2* was significantly increased than that of *matK* + *rbcL* in 18 families and 21 genera [20]. JIA *et al*. analyzed the biological composition of Yimu Wan and compared with four prescribed herbal materials by *ITS2* + *psbA-trnH* and single molecule real time sequencing (SMAT sequencing). The result showed SMART sequencing provided strong potential application to control the quality of traditional Chinese medicine patents [21].

Although many DNA barcoding studies were published, up to now, there were few literatures on species identification of poisonous herbal medicine. We still did not find a universal DNA barcode to identify poisonous medicinal plant. In this study, we collected all poisonous medicinal plants in Chinese pharmacopoeia (2015) and their adulterants or relative species to select a universal DNA barcode. Our findings showed that *ITS2* region should be a standard DNA barcode for the identification of poisonous medicinal plants in Chinese pharmacopoeia and their adulterants.

Materials and Methods

Poisonous plant species and candidate barcodes

We chose all poisonous medicinal plants in Chinese Pharmacopoeia (2015) and collected their adulterants or relative species including 106 species from 27 families and 65 genera, covering all different medicinal parts including root, whole grass, flower, fruits, seeds and cortex. Each species had 2−4 repeated samples. Basing on previous studies, our research chose four candidate barcodes (*ITS2*, *psbA-trnH*, *matK* and *rbcL*) to evaluate which region could be used as the standard barcode for poisonous medicinal plants.

PCR amplification and DNA barcoding analysis

Total DNA were isolated, amplified and sequenced in China Academy of Chinese Medical Sciences (CACMS). Sequences were submitted in GenBank after being assembled. The species name and GenBank accession numbers were shown in Supplementary Note 1 and the bold parts were the sequences uploaded to GenBank (SN 1 and 2).

The primers and PCR reaction conditions are applied according to CHEN *et al*. [18]. PCR products were sequenced bidirectionally using ABI 3730XL automated sequencer (Applied Biosysstems Inc). The genetic difference among all the species was analyzed by calculating both inter and intra Kimura 2-parameter (K2P) distance, and assessed by six distance value totally: average inter specific distance, average theta prime, average smallest inter-specific distance, average intra-specific distance, average theta, and average coalescent depth[22]. All the sequences used as DNA barcode were deleted gaps and aligned by MUSCLE default options and ClustalW in MEGA 6.0 to get sites information, construct generating K2P distance matrices for each locus and Neighbor-Joining (NJ) phylogenetic trees with bootstrap (1000 replications) respectively [MEGA Koichiro Tamura, Glen Stecher, Daniel Peterson, Alan Filipski, and Sudhir Kumar (2013)].

The identification efficiency of four candidate regions were evaluated using BLAST1 and nearest distance methods according to Ross *et al*. [23].

Results

Sequence alignment and analysis

Sequences obtained in this study and downloaded from Genbank (https://www.ncbi.nlm.nih.gov/) were aligned after data cutting. We noted sequence sites alignment information as described in Table 1.

The lengthes of four aligned regions were 218 bp, 388 bp, 700 bp and 910 bp respectively. Comparing these four regions,

the length of *ITS2* was significantly shorter than *psbA-trnH*, *matK* and *rbcL*. *ITS2* + *psbA-trnH* had high variable sites in percentage (88.99% + 84.79%) and singleton site in percentage (0.92% + 2.57%). In addition, *ITS2* had the highest C + G content (62.90%), the second was *rbcL* sequence (43.60%), only lower than *ITS2,* but it contained higher conserved sites in percentage (51.32%). The result showed that *rbcL* sequence was conserved relatively. *MatK* sequence had high variable sites in percentage (78.14%) and less singleton site in percentage (only 0.03%) compared with *ITS2* and *psbA-trnH*.

Table 1 Sequence alignment of *ITS2*, *psbA-trnH*, *MatK* and *RbcL*

Locus	Sequence Length in alignment(bp)	CS (%)	VS (%)	Pi (%)	SS (%)	C + G (%)
ITS2	218	11.01	88.99	88.07	0.92	62.90
psbA-trnH	388	12.63	84.79	81.70	2.58	28.06
MatK	700	19.57	78.14	74.86	0.03	31.85
RbcL	910	51.32	28.13	26.15	0.02	43.60

Sequence inter/intra-specific genetic distance and data distribution

We calculated inter/intra-specific genetic distances using Kimura's 2-Parameter sequence divergences by MEGA 6.0 software [MEGA Koichiro Tamura, Glen Stecher, Daniel Peterson, Alan Filipski, and Sudhir Kumar (2013)]. The results were shown in Table 2.

All the value of inter-specific distance was higher than intra-specific distance. Comparing both inter- and intra specific distance values, the 6 genetic distance values of *rbcL* were the lowest in the four tested barcodes. The average intra-specific distance was only 0.0010 ± 0.0029 matching with the characteristic that *rbcL* was conserved relatively. *ITS2* region exhibited higher variation with the maximum average inter-specific distance and average theta prime value, which were 0.6170 ± 0.1958 and 0.6417 ± 0.0998 respectively. Furthermore, we analyzed the data distribution of inter- and inra-specific genetic distance value (Fig. 1).

Table 2 Inter/intra-specific genetic distances (Kimura 2-parameter (K2P) distance [22]) basing on *ITS2*, *psbA-trnH*, *matK* and *rbcL*

Genetic distances	*ITS2*	*psbA-trnH*	*MatK*	*RbcL*
Average inter specific distance	0.6170 ± 0.1958	0.5687 ± 0.2512	0.5168 ± 0.3445	0.1409 ± 0.1798
Average theta prime	0.6417 ± 0.0998	0.6144 ± 0.2060	0.4709 ± 0.2562	0.1244 ± 0.1417
Average smallest inter-specific distance	0.2230 ± 0.1793	0.1963 ± 0.1980	0.1296 ± 0.1853	0.0204 ± 0.0260
Average intra-specific distance	0.0044 ± 0.0081	0.0056 ± 0.0126	0.0038 ± 0.0089	0.0010 ± 0.0029
Average theta	0.0047 ± 0.0083	0.0061 ± 0.0125	0.0047 ± 0.0087	0.0009 ± 0.0024
Average coalescent depthe	0.0061 ± 0.0104	0.0078 ± 0.0153	0.0066 ± 0.0113	0.0013 ± 0.0036

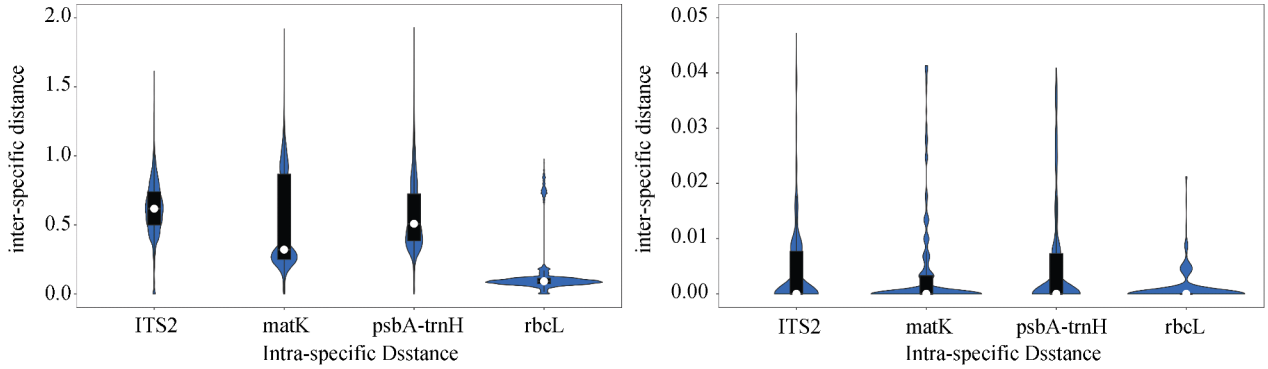

Fig. 1 The data distribution of inter/intra specific distance

The scale of *ITS2*, *psbA-trnH* and *matK* data span was wider than *rbcL*, especially for the scale of intera-specific distance distribution. Compared with *matK* and *rbcL*, both *ITS2* and *psbA-trnH* had similar and more uniform distribution scale of inter-/intra-specific distance value. Most intra-specific distance values were focused on 0−0.01, and inter-specific distance values of *ITS2*, *psbA-trnH* and *matK* were focused on 0.5−1.0, while all the genetic distance values of *rbcL* were lower than 0.5. For overall data distribution trend, genetic distance value scale of *ITS2* and *psbA-trnH* were in uniform relatively but *matK* and *rbcL* were more discretized, especially the average intra-specific distance value of *matK*.

Identification efficiency for ITS2, psbA-trnH, matK and rbcL

In order to select the most suitable barcode, we compared the identification efficiency of the four regions. All of the results were shown in Table 3.

In all taxa levels based on different analysis methods, the correct identification efficiency of *rbcL* was significantly lower than *ITS2*, *psbA-trnH* and *matK*. At species level, *ITS2*

had the same correct identification efficiency (92.59%) by both BLAST and distance method, which was close to the efficiency of distance methods at genus level (98.15%). Both *psbA-trnH* and *matK* had 100% correct identification efficiency at genus level, but lower than *ITS2* at species level. By general comparison, the correct identification efficiency values were *ITS2* > *psbA-trnH* > *matK* > *rbcL* at species level, and *psbA-trnH* = *matK* > *ITS2* > *rbcL* at genus level.

Table 3 Identification efficiency

Locus	Identification Method	Plant taxa level	Correct identification (%)	Incorrect identification (%)	Ambiguous identification (%)
ITS2	Blast	Species	92.59	0	7.41
		Genus	98.15	0	1.85
	Distance	Species	92.59	0	7.41
		Genus	98.15	0	1.85
psbA-trnH	Blast	Species	89.72	0	10.28
		Genus	100	0	0
	Distance	Species	83.18	0	16.82
		Genus	100	0	0
MatK	Blast	Species	85.98	0	14.02
		Genus	100	0	0
	Distance	Species	81.31	0	18.69
		Genus	100	0	0
RbcL	Blast	Species	75.70	0	24.30
		Genus	90.65	0	9.35
	Distance	Species	53.27	0	47.73
		Genus	79.44	0	20.56

Neighbor-Joining (NJ) phylogenetic trees

We constructed NJ phylogenetic trees basing on four regions and compared their distinguishing capability further according to the topological structure.

Among four NJ phylogenetic trees overall, in the topology of *ITS2,* most branches were independent either at either species or subspecies level. In *Liliaceae*, *Paris polyphylla*. var *chinensis* and *Paris polyphylla*. var. *yunnanensis* were divided into independent branches showing monophyletic characteristics. Every species belonging to the same genera was clustered into one group at family level. *PsbA-trnH*, *matK* and *rbcL* formed more single species fell outside their groups than *ITS2* at family level. And at species level, *Prunus* (belonging to *Rosaceae*) was easier to diverge in our samples. Additionally, *matK* and *rbcL* showed diagnostic shortcomings at species level, manifesting that closely related species in the same genus were clustered into one group, which were *Prunus dulcis* and *Prunus persica*, *Actaea dahurica* and *Actaea rubra*(*matK*), and *Uncaria macrophylla* and *Uncaria rhynchophylla* (*rbcL*).

Discussion and Conclusion

Plant poisoning is a common accident in our daily lives. At present, highly toxic plants can be distinguished from nontoxic ones according to simple toxidromic classification system [24]. DNA barcoding identifies species mainly depending on the difference among the particular sequences, which was used in differential diagnoses and directing earlier management of potentially serious plant ingestion. For both original plants and their productions, DNA barcoding method has been used to confirm the identity or purity [25].

This research aimed to find the most suitable DNA barcode to identify poisonous medical plants. We found both *ITS2* and *psbA-trnH* sequences showed higher genetic variability. And *ITS2* sequence has the shortest average length (218 bp) of all candidate barcodes. *RbcL* sequence was more conservative and less convenient to distinguish closely related species. It also exhibited similar results from all six specific distance values in genetic distance analysis (Table 2). Previous studies also supported that *ITS2* and *psbA-trnH* had the advantages of shorter sequences and higher variations [20].

By using BLAST and genetic distance methods, the correct identification efficiency was *ITS2* > *psbA-trnH* > *matK* > *rbcL* at species level and *psbA-trnH* = *matK* > *ITS2* > *rbcL* at genus level. Among all the candidate barcodes, *ITS2* exhibited highest correct identification efficiency using BLAST or distance method at species level (92.15%), and the result was consistent with the analysis data (92.7%) in the study of CHEN *et al*. (Table 3) [18].

MatK was previously considered as a suitable plant barcode due to its high evolutionary rate, suitable length, obvious inter-specific divergence and low transition/transversion rate. However, *matK* has a high substitution rate at the primer sites leading to the difficulty in amplification [26-27]. Comparing

with *ITS2* and *psbA-trnH*, *MatK* or *rbcL* was rejected as a universal barcode due to low PCR efficiency. *RbcL* is easy to be amplified, but loci was relatively conservative [28]. Osathanunkul *et al*. found that the CG% of *matK* and *rbcL* were 34.6%, 43.4% respectively in *Tinospora* species [29], which was similar to our results (31.85%, 43.60%) (Table 1). Francisco *et al*. found the CG content was close among related species [30]. According to our results, the CG content of four sequences was different. *ITS2* was a non-coding region with a conserved core of the secondary structure promoting the establishment of data handling system [31]. Ka-Lok Wong discovered that *matK* and *rbcL* could be used in the identification of related species of *Gentiana*, but did not show more advantages than other five sequences including *ITS* and *psbA-trnH* [17]. In the recent research of poisonous medical materials identification, *ITS2* combining with TLC and HPLC could identify *Marsdenia tenacissima* in the market [32]. And *psbA-trnH* showed high rates of insertion/deletion and most sequence divergence among non-coding intergenic regions [22]. According to the alignment of four barcodes, the percentage of singleton site in *psbA-trnH* was the highest (2.58%) and the following sequence was *ITS2* (0.92%) > *matK* (0.03%) > *rbcL* (0.02%) (Table 1).

Although the purpose of DNA barcoding is not intended to construct phylogenetic trees, the topology of NJ phylogenetic tree can evaluate identification efficiency of candidate barcodes. T. Orihara *et al*. discovered the phylogeographic relationships and evolutionary information of *Rossbeevera* (*Boletaceae*) by using three nuclear (*ITS*, *nLSU*, *EF-1α*) and two mitochondrial DNA sequences (*ATP6* and *mtSSU*) as well as precise morphological observation [33]. He highlighted the utility of *ITS* for molecular identification. At family level, the closer related species were in the same genus, the easier it was for *psbA-trnH*, *matK* and *rbcL* to form single specie divergences in this study, such as *Solanaceae*, *Araceae*, *Rutaceae* and *Ranunculaceae* (SFig. 2−4). And at species level, *matK* and *rbcL* region showed lower sequence divergences rates. The incorrect monophyletic branch percentage of *matK* and *rbcL* were 1.25% and 0.62% respectively. Comparing the cohesion of taxonomic groups of four candidate barcodes, all of the species in one family were clustered into one group only in the NJ phylogenetic trees of *ITS2*. In the branch of *Solanaceae*, samples of *Brugmansia arborea* formed an independent branch outside the group of *Datur*a, which was consistent with the result in the study of HAN *et al*. [5]. And *ITS2* had high cohesion and identification ability (SFig. 1).

A universe DNA barcode should have enough variability in the closely related species. For the taxon profiles of four NJ phylogenetic trees, the total percentage of single specie divergence was *rbcL* (51.09%) > *psbA-trnH* (49.82%) > *matK* (36.99%) > *ITS2* (18.31%). The conservation of *rbcL* may make it difficult to identify species with large intra-specific population variation accurately, as the analysis that *rbcL* is not suitable barcode at species level [22]. For example, overlapped characters affected the identification results of subdivisions of *Paris* by morphological methods [34]. We found *rbcL* and *matK* also could not form monophyletic branch of *Paris polyphylla* var. *chinensis* or *Paris polyphylla* var. *yunnanensis*. By contrast, *ITS2* showed distinct monophyletic assemblages for *Paris* signalling with more powerful diagnostic capability at subspecies level (SFig. 1). This was consistent with the findings of ZHU *et al*. [35]. *Rosaceae* only contained *Prunus* genus in our samples, but there were more single specie divergences at species level (SFig. 1−4). We inferred that the reason might be that the species have been bred by artificial cultivation form different cultivars (lines) or hybrids. The NJ phylogenetic trees formed single species fell outside their groups at species level in overall four barcodes. The single specie divergences percentage of *ITS2* was the lowest (2.03%), nearly 60% lower than *matK* (5.02%). XIE *et al*. conducted an analysis on the identification of poisonous plants by DNA barcoding and suggested that *ITS* combined *matK* and *rbcL* as a composite DNA barcode could increase discrimination rates using Blast method [36]. But the study could not provide a universe barcode for poisonous plans. In addition, *ITS* was not an ideal plant DNA barcode because of amplification problems [27].

By comparison, *ITS2* presented a good PCR efficiency and satisfactory identification efficiency in different classification levels. Moreover, *ITS2* exhibited rich spatial secondary structures which provided additional variation information for distinguishing closely related species. We believe that *ITS2* region should be considered a standard barcode for poisonous medicinal plants combining with *psbA-trnH* as its complementary barcode. On the other hand, medical materials are easy to be polluted by fungus. WANG *et al*. discovered a high fungal contamination rate (95%) in traditional Chinese medicine [37]. So in practice it is important to prevent fungal contamination in DNA extraction and PCR amplification when using *ITS2* as a suitable region for DNA barcoding applications [38].

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