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Molecular Authentication of Chinese Herbal Materials

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ABSTRACT

Traditional Chinese medicine (TCM) has been playing a major role in health care in China for millennia. Accurate authentication is always necessary to prevent the target herbs from intentional and inadvertent adulteration with other plant species. Morphological and histological authentication is now commonly practiced but they are not precise enough to authenticate those herbs which are possibly substituted or adulterated by plants with similar shapes and tissue constructs. Ordinary chemical authentication was also introduced to TCM but it is often not reliable enough to produce easy-to-interpret results. Therefore, it is necessary to develop a more effective, accurate, reliable and sensitive technology for the authentication of herbs. DNA manipulation techniques developed for molecular biotechnology have been adapted to the authentication of herbs in recent years. These techniques comprise of the molecular markers, sequencing of specific genes, and sophisticated hybridization setups such as DNA microarrays. Underside, we review the development of current techniques for authentication. In addition, we also describe the use of molecular markers in authentication of the most studied Chinese herbs.

Key words: molecular authentication, rDNA, Chinese herbal materials

INTRODUCTION

For thousands of years, traditional Chinese medicine (TCM) has been practiced and has played a crucial role in the health care and in helping the Chinese nation flourish. In spite of the great advances of modern medicine, TCM is still the primary form of healing methods for many people in Asia[(1)]. With its multi-target effects, TCM is particularly suitable for the treatment of modern diseases such as cardiovascular disease, asthma, and other long-term illnesses[(2)]. Furthermore, an increasing variety of healthy care products has been developed from TCM to meet the contemporary trend for "back to nature". Many famous multi-national medicine companies are now developing TCM jointly with Chinese companies. The World Health Organization has also been keen to pursue the development of TCM. The standardization and modernization of TCM depend on the authentication of the identity of Chinese medicinal materials. Therefore, the authentication and quality control have been the key for TCM to enter the world market[(3)].

Up till now, traditional morphological inspection is still widely used to distinguish the herbs. Morphological approach includes the inspection of shape, color, texture and odor of the herbs. For example, in the traditional authentication of Radix *Codonopsis*, the following criteria are used: the root is long cylindrical, slightly curved, 10-35 cm long and 0.4-2 cm in diameter[(4)] and the odour is characteristic, aromatic and tastes sweet[(5)]. The morphological inspection to authenticate TCM is simple and direct but its accuracy depends heavily on the examiners, which are sometimes subjective.

Histological techniques based on microscopic examinations are used to reveal the characteristics of tissue structure and arrangement in cork cell, cortex, sieve tubes, xylem vessels and cell components or content of a manufactured product. The thickness of the exodermal cell walls, diameter of exodermis, number of transfusion cells, and number of vascular bundles were used as markers for identifying the plants source of Herba Dendrobii[(6)]. The transverse section of Radix *Codonopsis* has over ten rows of cork cells. There are stone cells present at the outer side. The cortex is narrow[(7)]. Histological identification is not applicable to modern herbal drugs, for example, herbal capsules, troche and pills. Related species may share similar histological characteristics, making this approach not so accurate.

Chemical authentication emphasizes on the analysis of chemical constituents. Characteristic compositions are used for the differentiation. Thin layer chromatography (TLC) is the most common techniques to assess the chemical constituents of medicinal materials. For examples, TLC was used to identify *Tribulus terrestris*[(8)] and Fructus Xanthii[(9)]. High performance liquid chromatography (HPLC) has become the routine procedures to identify herbal materials. For example, HPLC has been used to analyze the chemical profiles of cassia bark (cortex *cinnamomi*)[(10)], to generate fingerprints for *Psoralea corylifolia*[(11)], to authenticate *Ephedra*[(12)] and to evaluate the quality of Radix Salviae Miltiorrhizae[(13)]. An accurate

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and reproducible reversed-phase HPLC was developed to determinate the content of atractylenoide III, which is known as the active constituent of *Codonopsis pilosula*[14]. Besides HPLC, other chemical approaches have also been developed including ultraviolet spectroscopy[15], infrared spectroscopy[16], gas chromatography/mass spectrometry[17], liquid chromatography/mass spectrometry[18] and liquid chromatography/mass spectrometry/mass spectrometry[19]. The compositions and relative amount of chemicals in a species may have also been developed with the growing conditions, harvesting periods, post-harvest processes and storage. The variation of chemical compositions may hinder the authentication, and in some instances, this can be misleading if the samples are deliberately adulterated with a marker compound. Moreover, it is difficult to distinguish closely related species due to similar chemical compounds.

I. *Molecular Markers for the Authentication of TCM*

(I) *DNA-based Molecular Markers*

DNA-based markers have now become a popular means for the identification and authentication of TCM from plants and animals. Major techniques include random amplified polymorphic DNA (RAPD)[20-23] and arbitrarily-primed polymerase chains reaction (AP-PCR)[24-25], polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[26-28], amplified fragment length polymorphism (AFLP)[29], direct amplification of length polymorphism (DALP)[30], specific sequence characterized amplified region (SCAR)[31-32] and short sequence repeat (SSR)[33]. Among these, RAPD technique was applied early to differentiate *C. pilosula* species from different areas in China. DNA fingerprints were used to distinguish Chinese herb Dangshen, the root of *C. pilosula* from different localities in China. This method may be applicable to locality authentication of other Chinese herbal materials (CHM)[34]. The phylogenic trees for *C. lanceolata* have been constructed for phylogenic analysis[35]. Genetic diversity, relationship and molecular authentication of total 8 wild populations of *Dendrobium officinale* were investigated using RAPD markers. Distinct genetic differences and extensive genetic diversity were presented among the wild populations. RAPD markers are informative and useful tools for the evaluation and authentication of wild populations of *D. officinale*[36]. Recently, RAPD was combined with other methods for the identification of Chinese medicines. For example, RAPD and Eastern blotting analyses using ginsenoside Rb1 and Rg1 monoclonal antibodies were employed to identify *Panax notoginseng*, *P. quinquefolius* and *P. japonicus*. RAPD was first used to differentiate the species of *Panax* spp. and thus the absence of ginsenoside Rc in the extract of *P. notoginseng* in the Eastern blot confirmed the identity of this species[37]. *Fritillaria pallidiflora* is a commonly used antitussive herb. The differentiation of eight *F. pallidiflora* species is limited by the current morphology-based and chemical methods. Therefore diagnostic PCR and PCR-RFLP have been established to differentiate *Fritillaria* species[38]. In Brazil, *Plectranthus* species are known as "boldo" and have been commonly used for analgesic and dyspeptic purposes. *Plectranthus* spp. need to be well identified in order to be used commercially and AFLP DNA patterns have been used to distinguish different *Plectranthus* species[39].

(II) *DNA Sequencing-based Markers*

DNA polymorphisms were studied by determining the nucleotide sequence in a defined region and aligning the sequence with homologous regions of related organisms[40]. This approach provides a highly reproducible and informative analysis and can be adapted to various levels of discriminatory potential by choosing appropriate regions of the genome. Currently, DNA sequencing is applied to distinguish species and study phylogenetic relationship, population genetics, systematics and evolution[41]. There are many reports concerning the application of DNA sequence-based markers to differentiate TCM from its substitutes or adulterants. Most of them involves the sequencing of internal transcribed spacer (ITS) ribosomal DNA (rDNA)[42-44], 5S rDNA gene[45-46], 18S rDNA and *trn*K genes[47], cytochrome b[48] and chloroplast DNA (cpDNA)[49]. First, the ITS rDNA region has become an important gene locus for the molecular systematic investigation of angiosperms at the interspecific and intraspecific levels. Specific PCR primers are positioned on the conserved rDNA genes (18S, 5.8S, 28S) to amplify the entire ITS spacer region (Figure 1). The ITS region of rDNA, defined as the unit containing the ITS1 spacer, 5.8S rDNA gene and ITS2 spacer, has been proven to be a useful gene for screening different species of TCM. ITS rDNA region is unable to differentiate *Codonopsis* species because the sequences are highly conserved[50]. On the other hand, the physical maps of chloroplast DNA (cpDNA) of *Codonopsis* genus were constructed and they are corresponding to the pollen morphology. It was suggested that cpDNA gene order mutations make an excellent phyloge-

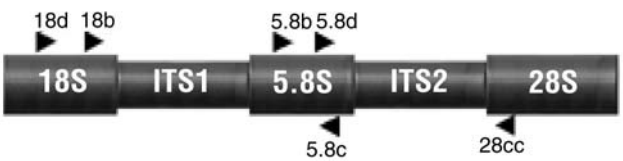

Figure 1. Schematic diagram of the nuclear rDNA internal transcribed spacer region. The three rDNA subunits: 18S, 5.8S and 28S are separated by internal transcribed spacers (ITS1 and ITS2). Arrows indicate the annealing sites of the primers used for PCR amplification. 18b: 5'-TAG AGG AGG GAG AAG TCG TA-3'; 18d: 5'-CAC ACC GCC CGT CGC TCC TAC CGA-3'; 28cc: 5'-ACT CGC CGT TAC TAG GGG AA-3'; 5.8b: 5'-TGA AGA ACG TAG CGA AAT GCG-3'; 5.8d: 5'-AAC CAT CGA GTC TTT GAA CGC A-3'.

netic marker[51]. Several laboratories have also employed ITS rDNA region for the authentication of *Dendrobium* species and its product Shihu. The sequences of ITS2 regions of 16 *Dendrobium* species differ from one another by an average of 12.4% and differ from non-orchids and *Pholidota* (an adulterant of *Dendrobium*) by 29.8% and 18.8%, respectively[52]. The ITS2 regions could be thus adopted as a molecular marker for differentiating medicinal *Dendrobium* species from one another and also from non-orchids and adulterants. The ITS sequences were also used to analyze a class of Herba Dendrobii with thin yellowish stems known as Huangcao Shihu[53]. There are two nucleotide differences in the ITS region between F type (can be processed to Fengdou Shihu) and H type (cannot be processed to Fengdou Shihu) of *D. officinale* in China[54]. The ITS region was used to authenticate Fengdou Shihu[55] and to distinguish *D. chrysanthum* from its relative species[56]. Further, the whole ITS1-5.8S-ITS2 rDNA regions of 28 *Dendrobium* species were sequenced (GenBank accession number AY485692-AY485719). The average difference of the ITS1 is 34.62% between *Dendrobium* and non-orchids, and 22.31% between the *Dendrobium* and the orchids; the interspecific difference among the *Dendrobium* species is 13.14% (Table 1), indicating that ITS1 may also be used to differentiate the concerned *Dendrobium* species[57]. Secondly, 5S rDNA gene of CHM was also explored. Radix Adenophorae (Shashen) is derived from the roots of *Adenophora stricta* and *A. tetraphylla*. Twelve species and varieties of *Adenophora* and *Glehnia*, however, have been used as substitutes or adulterants of Radix Adenophorae in the South East Asia markets. The 5S rDNA spacer domains (approximately 250 bp) were amplified by PCR from genomic DNAs from *A. stricta*, *A. tetraphylla*, *A. hunanensis* and *G. littoralis*, and sequences. The diversity in DNA sequences and restriction enzyme mapping among various species were found, which could serve as markers for the authentication of Radix Adenophorae[58]. *Rhizoma Curcumae* (Ezhu) has been used to remove blood stasis and to alleviate pain for centuries. The 5S rDNA spacer domains of five *Curcuma* species, including the common adulterants of this herb, were amplified and sequenced. The diversity in DNA sequenced was used for the quality control of these *Curcuma* species[59]. Thirdly, 18S, *trn*K, 12S, cytochrome b genes were also investigated. The six botanical origins of Chinese and Japanese *Curcuma* drugs were determined based on the comparison of 18S rDNA gene and *trn*K gene sequences. To develop a more convenient identification method, amplification-refractory mutation system (ARMS) analysis of both gene regions was performed. By the ARMS method, and the information on the region of production, the identification of *Curcuma* plants was achieved. The ARMS method for the *trn*K gene was also useful for authentication of *Curcuma* drugs[60]. Chloroplast *trn*K gene and nuclear 18S rDNA sequences of 13 *Panax* taxa, collected mainly from Sino-Japanese floristic regions, were investigated in order to construct the phylogenetic relationship and to assist the taxonomic delimitation within this genus[61]. To distinguish the Chinese crude drug Sailonggu (bone of plateau zokor, Myospalax baileyi) from its substitutes, two pairs of allele-specific diagnostic primers (SL1L/SL1H and SL2L/SL2H) were designed based on the mitochondrial 12S rDNA and cytochrome b genes sequences of the bamboo rat (*Rhizomys sinensis*) and black lipped pika (*Ochotona curzoniae*). Each of the two diagnostic primer pairs can be used to distinguish crude drug Sailonggu from its substitutes or adulterants. In addition, the results of sequence alignment and phylogenetic analysis are consistent with that of the relative-specific diagnostic PCR analysis[62]. Two regions inside the chloroplast *trn*K were selected for the authentication of *Atractylodes* Rhizome (Byaku-jutsu) and *A. Lancea* Rhizome (So-jutsu). By comparing the nucleotide sequence data sets, it is possible to discriminate Byaku-jutsu and So-jutsu and also to identify the original plant species of each crude drug specimen[63]. Last, Chloroplast chlB gene encoding the subunit B of light-independent protochlorophyllide reductase was amplified from herbarium and crude drug specimens of *Ephedra sinica*, *E. intermedia*, *E. equisetina*, and *E. przewalskii*, for the authentication of the corresponding crude drugs obtained in the Chinese market[64].

Table 1. The average percentage differences of the ITS1 rDNA gene among varies *Dendrobium*, orchids and non-orchids samples

Samples	Maximum (%)	Average (%)	Minimum (%)
Intraspecific	2	1.03	0
Interspecific	25	13.14	5
Other orchids	34	22.31	16
Non-orchids	43	34.62	31

(III) *DNA Microarray-based Makers*

At present, high-density miniatured microarrays (Biochip) have emerged as promising tools for the high throughput analysis of genomic data. DNA microarray revolutionizes the traditional way of one gene per experiment for the genome studies[65]. Armed with the ITS sequences, microarray of the ITS1-5.8S-ITS2 regions from 28 *Dendrobium* species, two other orchids and two non-orchids were generated. Distinctive hybridization profile showed that 24 *Dendrobium* species may be differentiated from one another. The differentiation of *D. officinale* and *D. hercoglossum*, *D. nobile* and *D. moniliforme* was achieved by 5S rDNA array. This work has shown that ITS microarray could be used not only to establish the identities of the various *Dendrobium* species, but also to authenticate the medicinal *Dendrobium* from adulterant orchids[54]. To develop a rapid, accurate and sensitive method for identifying the source plant from the product, ITS microarray was employed to authenticate the Herba Dendrobii from two medicinal formulations. In

this experiment, the ITS1-5.8S-ITS2 sequences were used as probes and the ITS2 sequences as target. The Herba Dendrobii in formulation A, which contained nine herbal materials, was found to be *D. nobile* (Figure 2). On the other hand, formulation B, which contains 12 components, was tested and the Herba Dendrobii in this formulation was found to be *D. lohohense*. The latter species in fact is not listed in the Chinese Pharmacopoeia and hence is a substitute of Herba Dendrobii[55]. These studies provide the very examples of DNA microarray technology in tracing a medicinal component from complex medicinal mixture. In addition, microarray technology has also been used to authenticate ginseng[66] and toxic traditional Chinese medicinal materials[67].

(IV) *The Limitation of Molecular Markers in Quality Control*

There are several limitations. Firstly, it is not easy to extract DNA from some medicinal materials using general methods, particularly for those processed species. Secondly, although the differentiation of the geographical origins by molecular markers, such as the chloroplast *mat*K gene sequence[68], ITS from nuclear rDNA[69], 18S rDNA gene[70] has been established from time to time, DNA markers may not correspond to the chemical profiles. Therefore, DNA markers together with the chemical fingerprint for quality control of CHM have been investigated. For example, three species of Rhizome *Curcuma* (Ezhu) including *C. wenyujin*, *C. phaeocaulis*, and *C. kwangsiensis* have been used as medicinal materials. Chemical components such as curdione, curcumol, and germacrone in the essential oil are considered as the active constituents in *R. Curcumae*. The amount of these chemicals varies among samples from different species or samples from the same species but from different regions of cultivation. Chemical fingerprints were generated from these species as the identification markers. At the same time, the 5S rDNA spacer domains of five *Curcuma* species, including the common adulterants of this herb, were sequenced. The chemical fingerprint together with the sequence data could serve as the marker for quality control of *Curcuma* species[59]. To identify the origin of *Panax notoginseng* and its seven adulterants, and to analyze *P. notoginseng* in different localities, the nuclear 18S rDNA and chloroplast genes were sequenced. HPLC fingerprinting was also used to correlate the chemical composition and geographical distribution. This study concluded that DNA markers can be applied to authenticate the easily-confused species and can help to trace their geographical origins[71]. For the quality evaluation of Pogostemon cablin cultivated in Guangdong and Hainan, two sequences, 1.2 kb of plastid *mat*K gene and 1.8 kb nuclear 18S rDNA gene, and two chemotypes (pogostone-type and Patchouliol-type in essential oil composition) were compared. The result showed that the sequence divergence in both *mat*K and 18S rDNA genes among six samples of *P. cablin* were well correlated with their regions of cultivation and intraspecific chemotypes of essential oil compositions[72]. Particularly, Chinese formulations noteworthy of multiple plant components make the identification more difficult, but it is not impossible. Testing for unknown contaminants is extremely difficult. These limitations are expected to be eliminated by advancement of molecular technology in the future.

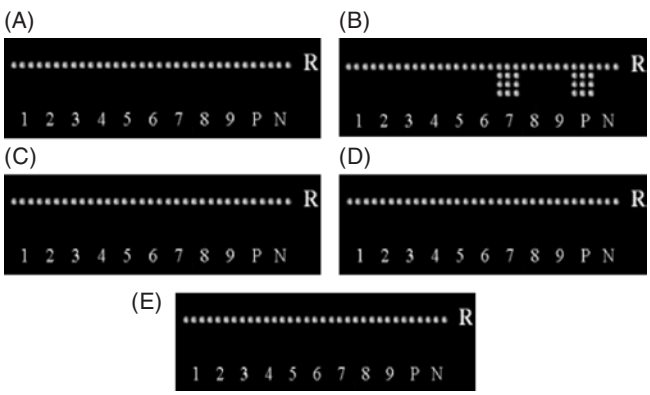

Figure 2. Microarray hybridization of individual herbal components in formulation A with Herba Dendrobii probe. Panels 1-9: spotted ITS-1-5.8S-ITS2 sequences of the herbs 1-9 in formulation A. Panels P and N: spotted ITS1-5.8S-ITS2 DNA from formulation A with or without Herba Dendrobii. Row R: spotted ITS1-5.8S-ITS2 DNA of the five *Dendrobium* species listed in the Chinese Pharmacopoeia. The result of hybridization with ITS2 rDNA from *D. fimbriatum* (A) *D. nobile* (B) *D. officinale* (C) *D. loddigesii* (D) and *D. chrysanthum* (E) respectively were showed.

CONCLUSIONS

Since the early paper concerning the differentiation of *P. quinguefolius* and *P. ginseng* by AP-PCR[20], many Chinese medicine materials have been authenticated by different molecular technologies (Table 2). Since more than 90% TCM uses either animal or plant as source material[73], molecular technologies may be an effective way to differentiate samples from different species or localities. Molecular markers have the advantages that they are least affected by age, environmental factors, and physiological conditions of the samples. These markers are not tissue-specific and thus can be detected at any stage of development. Moreover, a small amount of sample is sufficient for analysis and the physical form of the sample does not restrict the detection. These non-stringent requirements are particularly useful for some TCM that are expensive or in limited supply.

Urgent works for CHM quality control is to construct a comprehensive database of DNA fingerprints and DNA sequences for a broad spectrum of medicinal species. This comprehensive database containing voucher specimens, macro and microscopic data, chemical profiling and DNA fingerprinting information would clearly be beneficial for the authentication plant source, processing procedure, and

Table 2. List of Chinese medicines have been studies by different molecular technologies

Markers	TCM	Comments	Refs.
RAPD	*Glyrrhiza* species	Differentiation of four species	(74)
	Zaocys dhumnades	Identification of crude snake drugs	(75)
	Anoectochilus	Identification of two species	(76)
	Atractylodes plants	Revealed intraspecific variation	(77)
	Astragalus medicines	Differentiation of the two species	(78)
	Rabdosin serra plants	Authentication	(79)
	Amomun villosum species	Analysis of A. *villosum* and adulterants	(80)
	Scutellaria plants	Discrimination of the three species	(81)
	Panax notoginseng	Authentication of *P. notoginseng*	(82)
	Yu-ping-feng san	Identification of components	(83)
	Aconitum plants	Differentiation of *A. noveboracense* and *A. columbianum*	(84)
	Ginkgo biloba	Differentiation of the nine populations	(85)
RFLP	*Atractylodes lancea*	Revealed intraspecific variation	(86)
	Panax species	Differentiation of *P. ginseng* and *P. quinquefolius*	(87)
	Fritillaria pallidiflora	Identification	(88)
rbcL	*Belamcanda chinensis*	Analysis of *B. chinensis* and related plants	(89)
*trn*K	*Curcuma* drugs	Authentication	(90)
	Atractylodes drugs	Authentication of derived crude drugs	(91)
	Atractylodes plants	Phylogenetic analysis	(92)
*mat*K	*Panax vietnamensis*	Phylogenetic analysis	(93)
18S	*Panax notoginseng*	Analysis of homology	(94)
ITS	*Saussurea medusa*	Comparison on ITS sequences	(95)
	Herba Hedyotis Diffusae	Authentication	(96)
	Hypericum species	Genetic profiling	(97)
	Zanthexylum bungeanum Maxin	Authentication of population and adulterants	(98)
5S	*Fritillaria* species	Molecular diversity	(99)
	Radix Astragali	Species identification	(100)
	Ephedra plants	Phylogenetic analysis	(101)
12S	*Snake gallbladder*	Identification	(102)
Cyt b	*Oviductus ranae*	Authentication of original animals	(103)

for providing consumers with a safe product. The molecular detection technologies therefore undoubtedly contribute to the research and development of herbal drugs.

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