· Reviews ·

Authentication of Medicinal Plants by DNA-based Markers and Genomics

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Abstract: For the protection of consumers and developments of relevant industry, authentication of medicinal plants is a critical issue. This review covers various aspects of authentication methods and techniques based on molecular biology and genomics with special emphasis on molecular biology techniques including genome-based authentication, microchip-based authentication, DNA barcoding, and their applications.

Key words: authentication; DNA fingerprint; genotyping; medicinal plant; plant barcodes; traditional Chinese medicine
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Introduction

Botanical extracts, a new type of hi-tech product derived from modern pharmaceutical techniques, are not only the core products in the market of natural medicines, dietary supplements, and the raw materials of plant pharmaceutical reagents, but also used in the nutrient supplements and cosmetic products. In recent years, botanical extracts are accepted by the scientific community and approved by main stream markets. China's exports of traditional Chinese medicinal products increased by 10.27% compared to 2004 reaching a trade value of USD $153 million (Anonymous, 2006a; 2006b). In America, "Tropical Product" is a USDA category that includes herbs and spices, such as American ginseng root (*Panax quinquefolius* L.) and ginger rhizome (*Zingiber officinale* Roscoe), essential oil including peppermint leaf oil (*Mentha* × *piperita*) and spearmint leaf oil (*Mentha spicata* Linn.), herbal teas, as well as cocoa (*Theobroma cacao* L.) and coffee beans (*Coffea* spp.). U.S. exports of "Tropical Product" rose nearly 3% to USD $2.2 billion in 2005 (Brinckmann, 2006).

Whether they are being taken as dietary supplements by the general public or being evaluated in a clinical study, the authenticity of botanical products is a matter of paramount concern (Smillie and Khan, 2010). In order to protect consumers and promote development of the relevant industry, reliable authentication of plant materials is critically important. Authentication should be implemented in the whole process from the collection of the raw material to the finished products. In addition, they can be used to eliminate adulterants and fraudulent behavior of unscrupulous individuals. However, there is no single authentication method that can be applied to every medicinal plant. The chain of custody must be formed by combining various technologies and practices that are needed.

Traditionally the macroscopic and microscopic identifications are performed to authenticate plant materials at the species level. However, a staff's experience or training in plant identification may be limited. Moreover, morphology method is not suitable for the fragmented or powdered crude plant material. Plant metabolic profile and other physical-chemical properties determined by TLC, HPLC, IR, NMR, and

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X-ray (Mukherjee *et al*, 2010) are influenced by both genetic and environmental factors. Therefore, additional methods of identification at the species level have been sought and genome-based methods have been developed for the identification of medicinal plants starting in the early 1990's (Shaw, Wang, and But, 2002; Techen *et al*, 2004; Sucher and Carles, 2008; Hao, Yang, and Huang, 2009). This work was greatly facilitated by the invention of the polymerase chain reaction (PCR) and the introduction of a heat-stable DNA polymerase from the thermophilic bacterium. Presently, a practical and powerful tool, i.e., DNA barcodes, is developed for identifying medicinal plants and their adulterants in trade and for ensuring safety in their use (Chen *et al*, 2010; CBOL Plant Working Group, 2009). This review summarizes recent studies of our own and gives a comprehensive and prospective review of the implications of the authentication based on molecular biology and genomics, microchip-based authentication strategy, influencing factors and applications of molecular authentication, *etc*.

Molecular biology and genomics techniques for authentication

Amplified fragment length polymorphism PCR (AFLP) and restriction fragment length polymorphism (RFLP)

AFLP is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed in the early 1990's by Keygene (http://www.keygene.com), AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either through auto-radiography or fluorescence methodologies.

There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), RFLP, and microsatellites. AFLP not only has higher reproducebility, resolution, and sensitivity at the whole genome level compared to other techniques (Mueller and Wolfenbarger, 1999), but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt and Clarke, 2007). As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Passinho-Soares *et al* (2006), Wang *et al* (2008), Suo *et al* (2010) and Watthanachaiyingcharoen *et al* (2010).

RAPD and sequenced characterized amplified region marker (SCAR)

RAPD is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8−12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.

No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, though the exact binding site is unknown. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Because it relies on a large and intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize and trace the phylogeny of diverse plant and animal species. When developing locus-specific and co-dominant markers from RAPDs, new longer and specific primers are designed for the DNA sequence, which is called the SCAR. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Ruzicka *et al* (2009), Devaiah and Venkatasubramanian (2008), Cao *et al* (2010), and Choo *et al* (2009).

Inter-simple sequence repeat (ISSR) and simple sequence repeats (SSR)

Microsatellites, SSRs, or tandem repeats, are repeating sequences of 1－6 base pairs of DNA.

Microsatellites are typically neutral and co-dominant. They are used as molecular markers in genetics, for kinship, population, and other studies. ISSR is a general term for a genome region between microsatellite loci. The complementary sequences to two neighboring microsatellites are used as PCR primers; The variable region between them gets amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary much in length.

Sequences amplified by ISSR-PCR can be used for DNA fingerprinting. Since an ISSR may be a conserved or nonconserved region, this technique is not useful for distinguishing individuals, but rather for phylogeography analyses or maybe delimiting species; Sequence diversity is lower than in SSR-PCR, but still higher than in actual gene sequences. In addition, microsatellite sequencing and ISSR sequencing are mutually assisting, as one produces primers for the other. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Tamhankar *et al* (2009), Su *et al* (2008), Wang *et al* (2008), and Sharma *et al* (2008).

Arbitrarily primed (AP)-PCR and the direct amplification of length polymorphism (DALP)

AP-PCR is similar to RAPD but PCR is performed using sets of two longer primers (> 18 nucleotides) of arbitrary sequence. In DALP, PCR is conducted with variable forward primers that contain a universal core sequence at their 5' ends and a constant reverse primer resulting in multiple amplicons that can be separated by gel electrophoresis, isolated and directly sequenced. Examples of applications in the authentication of medicinal plants are seen in the papers of Ha *et al* (2001) and Cao, But, and Shaw (1996).

In the above approaches, rather than focusing on specific genetic loci, researchers make use of species-specific variations (polymorphisms) of the nucleotide sequence that are spread randomly over the entire genome resulting in characteristic "fingerprints" of genomic DNA. In the following approaches, the nucleotide sequences of one or more genetic loci ("genes") in the plants of interest are determined and the nucleotide sequences that are characteristic (i.e., inherited by all members) of a given species are identified (Table 1).

Multiplex PCR, PCR-SR

Multiplex PCR is a modification of PCR in order to rapidly detect deletions or duplications in a large gene. This process amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. Multiplex-PCR was first described in 1988 as a method to detect deletions in the dystrophin gene (Chamberlain *et al*, 1988). In 2008, multiplex-PCR was used for analysis of microsatellites and SNPs (Hayden *et al*, 2008).

Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. In PCR-selective restriction (SR), PCR amplicons obtained with gene specific primers are cut with restriction enzymes and analyzed by gel electrophoresis. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Jigden *et al* (2009; 2010) and Lin *et al* (2006).

Amplification refractory mutation system (ARMS) and Multiplex ARMS (MARMS)

ARMS is a variation of PCR that is based on the fact that the primers only bind to their target sequence when their 3' ends are complementary. Oligonucleotides with mismatched ("mutated") 3' end residues will not bind to the "normal" target sequences and no amplification will take place. MARMS is the multiplex PCR using a common primer and multiple mutation specific primers as used in ARMS. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Diao *et al* (2009), Qian *et al* (2008), and Ding *et al* (2008).

Melting curve analysis

Real-time PCR, also called quantitative real time PCR (Q-PCR/qPCR) or kinetic PCR, is a laboratory

technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. The information gathered can be used to infer the presence and identity of single-nucleotide polymorphisms (SNPs). Since the late 1990's product analysis via SYBR Green and other double-strand specific dyes, probe-based melting curve analysis had become nearly ubiquitous. The probe-based technique is sensitive enough to detect SNPs and can distinguish among homozygous wildtype, heterozygous, and homozygous mutant alleles by virtue of the dissociation patterns produced. Examples of recent applications in the authentication of medicinal plants are seen in the papers of Xue, Li, and Wang (2009) and Xue and Xue (2008).

Loop-mediated isothermal amplification (LAMP)

Studies of DNA polymorphisms in organisms have given us access to such information as possible diseases in humans, genomic evolution, and individual recognition. Many convenient methods to detect SNPs have been developed, including PCR-RFLP and TaqMan assay. In particular, LAMP (Sasaki and Nagumo, 2007) is convenient because the reaction could be conducted under isothermal conditions, thereby facilitating amplification. LAMP employs four kinds of primers and amplification proceeds only when all the primers are annealed to the target DNA. Although primer design is difficult because many combinations of primers are conceivable, amplification is achieved within two hours once a set of primers is designed. Moreover, it was reported that loop primers accelerated LAMP, achieving detection in less than one hour. An example of recent applications in the authentication of medicinal plants is seen in the paper of Sasaki, Komatsu, and Nagumo (2008).

Authentication of medicinal plants based on microchip

A DNA microarray is a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles (10−12 moles) of a specific DNA sequence, known as probes (or reporters). This can be a short section of a gene or other DNA elements that are used to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation.

DNA microarrays can be used to measure changes in expression levels, to detect SNPs, to genotype or resequence mutant genomes (Table 1). Microarrays also differ in fabrication, workings, accuracy, efficiency, and cost. Additional factors for microarray experiments are the experimental design and the methods of analyzing the data.

***5S ribosomal DNA* (*rDNA*)**

A silicon-based DNA microarray was designed and fabricated for the identification of toxic traditional Chinese medicinal plants (Carles *et al*, 2005). Species-specific oligonucleotide probes were derived from the *5S rRNA* gene of *Aconitum carmichaeli* Debx., *A. kusnezoffi* Reichb., *Alocasia macrorrhiza* (Linn.) Schott, *Croton tiglium* L., *Datura inoxia* Mill., *D. metel* L., *D. tatula* L., *Dysosma pleiantha* (Hance) Woodson, *D. versipellis* (Hance) M. Cheng ex Ying, *Euphorbia kansui* L., *Hyoscyamus niger* L., *Pinellia cordata* N.e. Brown, *P. pedatisecta* Schott, *P. ternata* (Thunb.) Breit., *Rhododendron molle* (Blum) G. Don, *Strychnos nux-vomica* L., *Typhonium divaricatum* (Linn.) Decne., and *T. giganteum* Engl., and the leucine transfer RNA gene of *A. pendulum* Busch and *Stellera chamaejasme* L. The probes were immobilized via dithiol linkage on a silicon chip. Genomic target sequences were amplified and fluorescently labeled by asymmetric PCR. Multiple toxic plant species were identified by parallel genotyping. Chip-based authentication of medicinal plants may be useful as an inexpensive and rapid tool for quality control and safety monitoring of herbal pharmaceuticals and neutraceuticals.

Sze *et al* (2008) constructed a DNA microarray for

Table 1 Strengths and weaknesses of the various authentication methods

Items	AFLP	RAPD	ISSR SSR	AP-PCR DALP	PCR-SR Multiplex PCR	ARMS MARMS
Basis of authentication	fingerprints of gDNA[1]	fingerprints of gDNA[1]	fingerprints of gDNA[1]	fingerprints of gDNA[1]	specific genetic loci	specific genetic loci
Cost	high	low	low	low	low	low
Time	slow	fast	fast	fast	fast	fast
Information content[2]	high	high	high	high	low	low
Multiplex ratio[3]	high	high	high	high	intermediate	low
Reproducibility	high	good[4]	good[4]	good	good	good
Accuracy	high	good	good	good	good	good
Database	none	none	none	none	none	none
Identification level	up to intra-specific; population	up to intra-specific; population	up to intra-specific; population	up to intra-specific; population	species	up to strain
Effectiveness in assessing relationships btw accessions	effective	Effective	Effective	Effective	Not applicable	Effective
Prior sequence knowledge	no	no	no	no	yes	yes
Level of skills required	medium	low	low-medium	low	low	low
Automation	yes	yes	yes	yes	yes	yes
Possible problems; limitations	radioactive contamination; high template purity	markers are dominant[7]	null alleles[8]	sensitive to reaction conditions; quality, quantity and purity of templates	positive controls needed; PCR inhibitory effects	

Items	LAMP	melting curve qPCR	Microchip Microarray	DNA barcoding
Basis of authentication	specific genetic loci	specific genetic loci	specific genetic loci	specific genetic loci
Cost	low	high	high	intermediate
Time	fast	fast	slow	fast
Information content[2]	low	low	high	low
Multiplex ratio[3]	low	low	high	low
Reproducibility	good	good	intermediate	Good
Accuracy	good	good	good	Good
Database	none	none	none	barcode of life[5] biobarcode[6]
Identification level	species	species	up to subspecies	species
Effectiveness in assessing relationships btw accessions	not applicable	not applicable		
Prior sequence knowledge	yes	yes	yes	no
Level of skills required	high	high	medium	medium
Automation	yes	yes	difficult	yes
Possible problems; limitations	susceptible to PCR inhibition; primer design	data difficult to exchange[9] statistical challenges[10]	groups with little sequence diversity; Resolution of recently diverged species; Hybrids	

1: species-specific variations (polymorphisms) of the nucleotide sequence

2: expected heterozygosity

3: No. of loci simultaneously analyzed per expt, throughput

4: only if SOPs are in place and the same commercial PCR mixes are used

5: http://www.barcoding.si.edu/dnabarcoding.htm

6: http://www.asianbarcode.org

7: not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies)

8: microsatellites fail to amplify in PCR

9: due to the lack of standardization in platform fabrication, assay protocols, and analysis methods

10: include taking into account effects of background noise and appropriate normalization of the data

high throughput identification of the plant resource of commercial FDSH [*Fengdu Shihu* (*Dendrobium officinale* Kimura et Migo)]. The 5S rDNA intergenic spacer region in *D. officinale*, *D. nobile* Lindl., *D. moniliforme* (L.) Sw., *D. hercoglossum* Rchb.f., *D. williamsonii* Day et Rchb. F., *D. capillipes*, *D. wilsonii*, and *D. jenkinsii* was amplified by a single primer pair and sequenced. The sequences showed polymorphism. They were incorporated on a glass slide and hybridized with fluorescently labelled 5S sequences from commercial *Shihu*. The DNA microarray enabled the differentiation of *D. officinale* from the other species tested. FDSH could thus be distinguished from its adulterants. It is evident that DNA microarrays provide a high-throughput and reliable approach for the identification of plant resources, and the method is useful for the authenti- cation of FDSH.

18S rDNA

Ginseng drugs, derived from underground parts of *Panax* species (Araliaceae), are the most important group of herbal medicines in the Orient. Previously, the nucleotide sequences of the nuclear *18S rRNA* gene of 13 *Panax taxa* were determined, as were the specific polymorphic nucleotides for identification of each species. On the basis of the nucleotide difference, a DNA microarray (PNX array) was developed for the identification of various *Panax* plants and drugs (Zhu, Fushimi, and Komatsu, 2008). Thirty-five kinds of specific oligonucleotide were designed and synthesized as probes spotting on a decorated glass slide, which included 33 probes corresponding to the species-specific nucleotide substitutions and 2 probes as positive and negative controls. The species-specific probes were of 23−26 bp in length, in which the substitution nucleotide was located at the central part. Triplicate probes were spotted to warrant accuracy by correcting variation of fluorescent intensity. Partial *18S rRNA* gene sequences amplified from *Panax* plants and drugs as well as their derived health foods were fluorescently labeled as targets to hybridize to the PNX array. After hybridization under optimal condition, specific fluorescent patterns were detected for each *Panax* species, and the analyzed results could be indicated as barcode patterns for quick distinction. The developed PNX array provided an objective and reliable method for the authentication on plants of *Panax* L. and drugs as well as their derived health foods.

26S rDNA

Tsoi *et al* (2003) investigated the genetic polymorphism of several species of *Fritillaria* L. and developed a DNA chip for the genotyping and identification of the origin of various species of *Fritillaria* L. at molecular level. Genomic DNA (gDNA) from bulbs of several species of *Fritillaria* L. was extracted and the polymorphisms of the D2 and D3 regions inside the *26S rDNA* gene were identified by direct sequencing. Oligonucleotide probes specific for these polymorphisms were designed and printed on the poly-lysine coated slides to prepare the DNA chip. PCR products from the species of *Fritillaria* L. were labeled with fluorescence by incorporation of dye-labeled dideoxy-ribonucleotides and hybridized to the immobi-lized probes on the chip. The polymorphisms were used as markers for discrimination among various species. Differentiation of the various species of *Fritillaria* L. was accomplished based on hybridization of fluorescent labeled PCR products with the DNA chip. The results demonstrated the reliability of using DNA chips to identify different species of *Fritillaria* L., and the DNA chip technology can provide a rapid and high throughput tool for genotyping and quality assurance of the plant species verification.

Subtracted diversity array (SDA)

Traditionally, the identification of plants relied on conventional techniques, such as morphological, anatomical, and chemical profiling, that are often inefficient or unfeasible in certain situations. Extensive literature exists describing the use of PCR DNA-based identification techniques, which offer a reliable platform, but their broad application is often limited by a low throughput. However, hybridization-based microarray technology represents a rapid and high-throughput tool for genotype identification at the molecular level. Using an innovative technique, an SDA of 376 features was constructed from a pooled gDNA library of 49 angiosperm species, from which the pooled nonangiosperm gDNA was subtracted (Jayasinghe *et al*, 2007). The SDA method was superior in accuracy, sensitivity and efficiency, and showed high-throughput capacity and broad application. SDA was validated for potential genotyping use, and the

results indicated a successful subtraction of non-angiosperm DNA. Statistical analysis of the polymorphic features from the pilot study enabled the establishment of accurate phylogenetic relationships, confirming the potential use of the SDA technique for genotyping. Further, the technique substantially enriched the presence of polymorphic sequences; 68% were polymorphic when using the array to differentiate six angiosperm clades (Asterids, Rosids, Caryophyllids, Ranunculids, Monocots, and Eumagnoliids). The "proof of concept" experiments demonstrate the potential of establishing a highly informative, reliable and high-throughput microarray-based technique for novel application to sequence independent genotyping of major angiosperm clades.

The accurate identification of medicinal plants is becoming increasingly important due to reported concerns about purity, quality, and safety. SDA was thus validated for the ability to distinguish clade-level targets in a phylogenetically accurate manner. Jayasinghe *et al* (2009) further investigated the SDA for genotyping capabilities, including the genotyping of plant species which was not included during the construction of the SDA, as well as to lower classification levels including family and species. The results show that the SDA has the ability to accurately genotype species which was not included during SDA development to clade level. Additionally, for those species that were included during SDA development, genotyping is successful to the family level, and to the species level with minor exceptions. Twenty polymorphic SDA features were sequenced in a first attempt to characterize the polymorphic DNA between species, which showed that transposon-like sequences may be valuable as polymorphic features to differentiate angiosperm families and species. Future refinements of the SDA to allow more sensitive genotyping are discussed with the overall goal of accurate medicinal plant identification in mind.

PCR-short tandem repeats (STR)

A microchip electrophoresis method coupled with the STR technique was developed for rapid authentication of ginseng species (Qin *et al*, 2005). A low viscosity hydroxypropyl methylcellulose solution was used as the sieving matrix for separation of the amplified STR fragments. The allele sizing of the amplified PCR products could be detected within 240 s or less. Good reproducibility and accuracy of the fragment size were obtained with the relative standard deviation for the allele sizes less than 1.0% (n = 11). At two microsatellite loci (CT 12 and CA 33), American ginseng had a different allele pattern on the electropherograms compared with that of the Oriental ginseng. Moreover, cultivated and wild American ginseng can be distinguished on the basis of allele sizing. This work establishes the feasibility of fast genetic authentication of ginseng species by use of microchip electrophoresis.

Molecular basis of genome-based authentication: molecular phylogenetics/DNA barcoding

Molecular phylogenetics, also known as molecular systematics, is the use of the structure of molecules to gain information on an organism's evolutionary relationships. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree. The most common approach is the comparison of homologous sequences for genes using sequence alignment techniques to identify similarity. Another application of molecular phylogeny is in DNA barcoding, where the species of an individual organism is identified using small sections of chloroplast/nuclear/mitochondrial DNA. The use of genome-based methods for the authentication of medicinal plants should be seen in the context of plant phylogenetic studies and a general effort aimed at barcoding of all plants. For example, we contribute to the phylogeny and specific delineation of the two conifer families, Taxaceae and Cephalotaxaceae, on the basis of molecular data (Hao *et al*, 2008). A cladistic analysis of the sequences of five chloroplast (*matK*, *rbcL*, *trnL*, *trnL-trnF*, and *psbA-trnH* spacer) and one nuclear (ITS) molecular marker was carried out, both individually and in combination, by distance, parsimony, likelihood, and Bayesian methods. The results confirm that the two families are monophyletic. Phylogenetic relationships were clarified in the respective genus, which contains many important medicinal plants (e.g., the source plant of taxane). *psbA-trnH*, *rbcL* third codon position, and *matK* first codon position contributed most to the separation of taxa in discriminant function analysis. Our results confirm, on a basis of multiple molecular markers and a complete sampling of basic species, the suggested

monophyly of Taxaceae and Cephalotaxaceae and propose interspecific relationships within each group, with profound nomenclatural, taxonomic, and authentication implications.

Hao, Chen, and Xiao (2009) analyzed the evolutionary patterns of sequence divergence in genes from the medicinal genus *Taxus* L., encoding paclitaxel biosynthetic enzymes taxadiene synthase (TS) and 10-deacetylbaccatin III-10 beta-*O*-acetyltransferase (DBAT). *N*-terminal fragments of *TS*, full-length *DBAT* and ITS were amplified from 15 closely related species of *Taxus* L. and sequenced. Premature stop codons were not found in *TS* and *DBAT* sequences. Codon usage bias was not found, suggesting that synonymous mutations are selectively neutral. *TS* and *DBAT* gene trees are not consistent with the ITS tree, where species formed monophyletic clades. In fact, for both genes, alleles were sometimes shared across species and parallel amino acid substitutions were identified. While both *TS* and *DBAT* are, overall, under purifying selection, we identified a number of amino acids of TS under positive selection based on inference using maximum likelihood models. Positively selected amino acids in the N-terminal region of TS suggest that this region might be more important for enzyme function than previously thought. Moreover, we identify lineages with significantly elevated rates of amino acid substitution using a genetic algorithm. These findings demonstrate that the pattern of adaptive paclitaxel biosynthetic enzyme evolution can be documented between closely related species of *Taxus* L., where species-specific taxane metabolism has evolved recently. In another study, a cladistic analysis of *Taxus*, using the sequences of one chloroplast (*trnS-trnQ* spacer) and three nuclear *TS*, *DBAT*, and *18S rDNA* markers, was carried out by distance, parsimony, likelihood, and Bayesian methods (Hao, Huang, and Yang, 2008). Three of the four New World species (*T. brevifolia*, *T. floridana*, and *T. globosa*) form a well-supported clade, whereas *T. canadensis* initially branches — appearing distantly related to both Old World taxa and New World species. In Asia, *Taxus chinensis*, *T. mairei*, *T. sumatrana*, and *T. wallichiana* cluster together and are sisters to a clade containing *T. baccata* and *T. contorta*. *Taxus yunnanensis* is more closely related to *T. wallichiana* than other four *Taxus* species from China; *T. contorta* is closer to the Euro-Mediterranean *T. baccata* than the Asian species. This study provides a genetic method for authentication of economically important *Taxus* species and proposes a robust phylogenetic hypothesis for the genus. Using *trnS-trnQ* spacer sequences, we were able to distinguish *T. mairei* (the major source plant of taxane) from all other species of *Taxus*.

DNA barcoding is the dominant method for species identification and discovery (Table 1). All other methods have various limitations and can not be used in a large scale and an efficient manner. As a result, all new researchers should focus on DNA barcoding technology. DNA barcoding uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification (Kress *et al*, 2005). Although barcodes are sometimes used in an effort to identify unknown species or assess whether species should be combined or separated, such usage, if possible at all, pushes the limits of what barcodes are capable of (Seberg and Petersen, 2009).

Applications include, for example, identifying plant leaves even when flowers or fruits are not available, identifying the diet of an animal based on stomach contents or feces, and identifying products in commerce (e.g., herbal supplements or wood; Kress *et al*, 2005). DNA barcoding is a novel technology that uses a standard DNA sequence to facilitate species identification. Although a consensus has not been reached regarding which DNA sequences can be used as the best plant barcodes, the *psbA-trnH* spacer region has been tested extensively in recent years.

Yao *et al* (2009) hypothesized that the *psbA-trnH* spacer regions were effective barcodes for species of *Dendrobium* Sw. The chloroplast *psbA-trnH* intergenic spacers of 17 species of *Dendrobium* Sw. were sequenced. The sequences were found to be significantly different from those of other species, with percentages of variation ranging from 0.3% to 2.3% and an average of 1.2%. In contrast, the intraspecific variation among the species of *Dendrobium* Sw. studied ranged from 0% to 0.1%. The sequence difference between the *psbA-trnH* sequences of 17 species of *Dendrobium* Sw. and one *Bulbophyllum odoratissimum*

ranged from 2.0% to 3.1%, with an average of 2.5%. The *psbA-trnH* intergenic spacer region could be used as a barcode to distinguish various species of *Dendrobium* Sw. and to differentiate species of *Dendrobium* Sw. from other adulterating species. Medicinal plants belonging to the family Polygonaceae in *Chinese Pharmacopoeia* possess important medicinal efficacy in traditional Chinese medicines. DNA barcodes are for the first time used to discriminate the Polygonaceae in *Chinese Pharmacopoeia* and their adulterants (Song *et al*, 2009). DNA samples, extracted from thirty-eight specimens belonging to eighteen species in Polygonaceae, were used as templates. Eight candidate barcodes were amplified by PCR. Species identification was performed using MEGA V 4.0. The amplification efficiency of six candidate DNA barcodes (*rbcL*, *trnH-psbA*, *ndhJ*, *rpoB*, *rpoC1*, and *accD*) was 100%, while the efficiencies of YCF5 and nrITS were 56% and 44%, respectively. The interspecific divergence was highest for the *trnH-psbA* (20.05%), followed by the nrITS (14.01%) across all species pairs, while intraspecific variation both within populations and between populations was absent. The *trnH-psbA* can not only distinguish ten species of Polygonaceae in *Chinese Pharmacopoeia*, but also recognize eight other species of Polygonaceae including their adulterants.

The *psbA-trnH* intergenic region is among the most variable regions in the gymnosperm chloroplast genome (Hao, Chen, and Xiao, 2010c). It is proposed as suitable for DNA barcoding studies and is useful in species level phylogenetics. This region consists of two parts differing in their evolutionary characteristics: 1) the *psbA* 3′ UTR (untranslated region) and 2) the *psbA-trnH* intergenic spacer. We compared the sequence and RNA secondary structure of the *psbA* 3′ UTR across gymnosperms and found consensus motifs corresponding to the stem portions of the RNA stem-loop structures and a consensus TGGATTGTTATGT box. The *psbA-trnH* spacer is highly variable in length and composition. Tandem repeats that form stem–loop structures were detected in both the *psbA* 3′UTR and the *psbA-trnH* spacer. The topology of the *psbA-trnH* tree may reflect the suitability of this marker for DNA barcoding in Taxaceae, but not in Cephalotaxaceae. *psbA-trnH* could not be candidate marker of DNA barcoding for Ephedrales (Fig. 1A) and Araucariaceae (Fig. 1B), but could be suitable for Cupressaceae and Pinaceae (Fig. 1C). Whether it is valid for Gnetales and Podocarpaceae is worth further study. The *psbA-trnH* spacer primers specified by Kress *et al* (2005) yielded distinct double bands in all Cycadales species but *Cycas* (Sass *et al*, 2007), and the addition of *psbA-trnH* sequence data did not further resolve the non-specific identification made by nrITS for the species tested. This is in accordance with our finding that *psbA-trnH* might not be used as the barcoding marker of Cycadales (Fig. 1B).

The plant working group of the Consortium for the Barcode of Life recommended the two-locus combination of *rbcL+matK* as the plant barcode, yet the combination was shown to successfully discriminate among 907 samples from 550 species at the species level with a probability of 72% (CBOL Plant Working Group, 2009). The group admits that the two-locus barcode is far from perfect due to the low identification rate, and the search is not over. Chen *et al* (2010) compared seven candidate DNA barcodes (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS) from medicinal plant species. The ranking criteria included PCR amplification efficiency, differential intra- and inter-specific divergences, and the DNA barcoding gap. It was found that the second internal transcribed spacer (ITS2) of nuclear rDNA represents the most suitable region for DNA barcoding applications. Furthermore, Chen *et al* (2010) tested the discrimination ability of ITS2 in more than 6600 plant samples belonging to 4800 species from 753 distinct genera and found that the rate of successful identification with the ITS2 was 92.7% at the species level. The ITS2 region can be potentially used as a standard DNA barcode to identify medicinal plants and their closely related species. ITS2 could serve as a novel universal barcode for the identification of a broader range of plant taxa. For example, Gao *et al* (2010) found that ITS2 sequences had considerable variation at the genus and species level within the family Fabaceae. The intra-specific divergence ranged from 0% to 14.4%, with an average of 1.7%, and the inter-specific divergence ranged from 0% to 63.0%, with an average of 8.6%. Twenty-four species found in the *Chinese Pharmacopoeia*, along with other 66 species including their adulterants, were successfully identified based on ITS2 sequences. In addition, ITS2 worked well, with over 80.0% of species

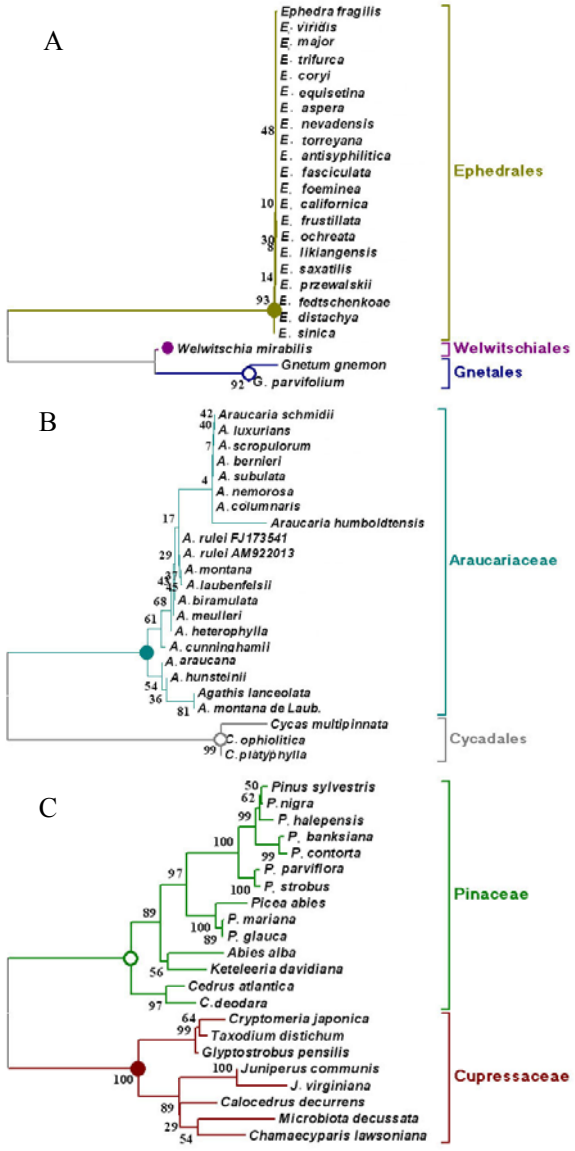

Fig. 1 Phylogenetic relationship of nucleotide sequences of gymnosperm *psbA-trnH* intergenic regions revealed by neighbour joining (NJ) trees

NJ used maximum composite likelihood (MCL) distances and pairwise deletion of gaps. Numbers beside the branches are bootstrap values. A: Gnetales, Welwitschiales, and Ephedrales; B: Araucariaceae and Cycadales; C: Cupressaceae and Pinaceae

and 100% of genera being correctly differentiated for the 1507 sequences derived from 1126 species belonging to 196 genera. Pang *et al* (2010) tested the applicability of four DNA regions (RBCL, MATK, ITS, and ITS2) as barcodes for identifying species within Euphorbiaceae. Based on assessments of the specific genetic divergence, the DNA barcoding gap, and the ability for species discrimination, the results affirmed that ITS/ITS2 is a potential barcode for the Euphorbiaceae species. This study also provided a large-scale test to evaluate the effectiveness of ITS/ITS2 for differentiating species within Euphorbiaceae. Of the 1183 plant samples collected from 871 species in 66 diverse genera, ITS/ITS2 successfully identified > 90% and 100% of them at the species and genus levels, respectively. Therefore, use of the ITS/ITS2 region is a powerful technique for Euphorbiaceae identification. In addition, the molecular evolution and sequence characteristics of the important barcoding markers such as *matK*, *rbcL*, and *trnL-trnF* spacer were also studied in detail (Hao, Huang, and Chen, 2009; Hao, Chen, and Xiao, 2009; 2010a; 2010b).

Conclusion

A large number of molecular techniques have been used to authenticate medicinal plants based on species-specific variations in the sequences of various chloroplast and nuclear DNA regions (Table 1). Genomic fingerprinting can differentiate between individuals, species and populations and has been proved useful for the characterization of sample homogeneity and detection of adulterants. DNA-based authentication of medicinal plants is a work in progress that offers powerful new tools and entry points for measures aimed at quality control and quality assurance in medicinal plant research as well as the production, clinical use, and forensic examination of herbal medicines. However, a few failure cases were reported. It is necessary to examine the failure rate of various DNA methods, especially for those high-value medicinal plants. It should be noted that DNA-based species identification alone is not sufficient for quality control and assurance because plants are the products of both the genome and the environment. The chemical metabolites play an important role in mediating the pharmacologic effects of herbal medicines, therefore the importance of extensive and standardized phytochemical characterization of medicinal plants by chromatographic and spectroscopic methods cannot be overemphasized. A complete array of authentication and evaluation tools can be utilized to provide a well-rounded scientific approach to the authentication of medicinal plants and botanical products.

References

Anonymous, 2006a. TCM Exports Up 10.27% in 2005. Xinhua News Agency.

Anonymous, 2006b. China's exports of medical products reach 13.8 billion US dollars. People's Daily Online.

Brinckmann J, 2006. ITC Consultant, MNS Medicinal Plant and Extracts report.

Cao H, But PP, Shaw PC, 1996. Authentication of the Chinese drug "ku-di-dan" (*Herba Elephantopi*) and its substitutes using random-primed polymerase chain reaction (PCR). *Acta Pharm Sin* 31: 543-553.

Cao L, Li SX, Wei BY, Huang D, Xu F, Tong ZY, 2010. Optimizing RAPD reaction system and authentic genuineness related genetic background of *Fructus Evodia*. *Chin Tradit Herb Drugs* 41(6): 975-978.

Carles M, Cheung MK, Moganti S, Dong TT, Tsim KW, Ip NY, Sucher NJ, 2005. A DNA microarray for the authentication of toxic traditional Chinese medicinal plants. *Planta Med* 71: 580-584.

Carles M, Lee T, Moganti S, Lenigk R, Tsim KW, Ip NY, Hsing IM, Sucher NJ, 2001. Chips and Qi: Microcomponent-based analysis in traditional Chinese medicine. *Fresenius J Anal Chem* 371: 190-194.

CBOL Plant Working Group, 2009. A DNA barcode for land plants. *Proc Natl Acad Sci USA* 106: 12794-12797.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT, 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res* 16: 11141-11156.

Chen SL, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y, Leon C, 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* 5: e8613.

Choo BK, Moon BC, Ji Y, Kim BB, Choi G, Yoon T, Kim HK, 2009. Development of SCAR markers for the discrimination of three species of medicinal plants, *Angelica decursiva* (*Peucedanum decursivum*), *Peucedanum praeruptorum* and *Anthricus sylvestris*, based on the internal transcribed spacer (ITS) sequence and random amplified polymorphic DNA (RAPD). *Biol Pharm Bull* 32: 24-30.

Devaiah KM, Venkatasubramanian P, 2008. Genetic characterization and authentication of *Embelia ribes* using RAPD-PCR and SCAR marker. *Planta Med* 74: 194-196.

Diao Y, Lin XM, Liao CL, Tang CZ, Chen ZJ, Hu ZL, 2009. Authentication of *Panax ginseng* from its adulterants by PCR-RFLP and ARMS. *Planta Med* 75: 557-560.

Ding G, Zhang D, Feng Z, Fan W, Ding X, Li X, 2008. SNP, ARMS and SSH authentication of medicinal *Dendrobium officinale* KIMURA et MIGO and application for identification of Fengdou drugs. *Biol Pharm Bull* 31: 553-557.

Gao T, Yao H, Song J, Liu C, Zhu Y, Ma X, Pang X, Xu H, Chen S, 2010. Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2. *J Ethnopharmacol* 130: 116-121.

Ha WY, Yau FC, But PP, Wang J, Shaw PC, 2001. Direct amplification of length polymorphism analysis differentiates *Panax ginseng* from *P. quinquefolius*. *Planta Med* 67: 587-589.

Hao DC, Chen SL, Xiao PG, 2009a. Molecular evolution and positive Darwinian selection of the chloroplast maturase *matK*. *J Plant Res* 123: 241-247.

Hao DC, Chen SL, Xiao PG, 2009b. Authentication of medicinal plants based on molecular biology and genomics. *Chinese J Pharm Biotech* 16: 490-494.

Hao DC, Chen SL, Xiao PG, 2010a. Physicochemical evolution and positive selection of the gymnosperm matK proteins. *J Genet* 89: 81-89.

Hao DC, Chen SL, Xiao PG, 2010b. Molecular evolution and positive Darwinian selection of the gymnosperm photosynthetic Rubisco enzyme. *Bota Studi* 51: 4.

Hao DC, Chen SL, Xiao PG, 2010c. Sequence characteristics and divergent evolution of the chloroplast *psbA-trnH* noncoding region in gymnosperms. *J Appl Genet* 51: 259-273.

Hao DC, Huang B, Chen SL, 2009. Evolution of the chloroplast *trnL-trnF* region in the gymnosperm lineages Taxaceae and Cephalotaxaceae. *Biochem Genet* 47: 351-369.

Hao DC, Huang B, Yang L, 2008. Phylogenetic relationships of the genus *Taxus* inferred from chloroplast intergenic spacer and nuclear coding DNA. *Biol Pharm Bull* 31: 260-265.

Hao DC, Xiao PG, Huang B, Ge G, Yang L, 2008. Interspecific relationships and origins of Taxaceae and Cephalotaxaceae revealed by partitioned Bayesian analyses of chloroplast and nuclear DNA sequences. *Plant Syst Evol* 276: 89-104.

Hao DC, Yang L, Huang B, 2009. Molecular evolution of paclitaxel biosynthetic genes *TS* and *DBAT* of *Taxus* species. *Genetica* 135: 123-135.

Hayden MJ, Nguyen TM, Waterman A, Chalmers KJ, 2008. Multiplex-ready PCR: A new method for multiplexed SSR and SNP genotyping. *BMC Genomics* 9: 80.

Jayasinghe R, Hai Niu L, Coram TE, Kong S, Kaganovitch J, Xue CC, Li CG, Pang EC, 2009. Effectiveness of an innovative prototype subtracted diversity array (SDA) for fingerprinting plant species of medicinal importance. *Planta Med* 75: 1180-1185.

Jayasinghe R, Kong S, Coram TE, Kaganovitch J, Xue CC, Li CG, Pang EC, 2007. Construction and validation of a prototype microarray for efficient and high-throughput genotyping of angiosperms. *Plant Biotechnol J* 5: 282-289.

Jigden B, Wang H, Kyum Kim M, Kim YJ, Gyo In J, Yang DC, 2009. Authentication of the oriental medicinal plant *Ligusticum tenuissimum* (Nakai) Kitagawa (Korean Go-Bon) by Multiplex PCR. *Planta Med* 76: 648-651.

Jigden B, Wang H, Samdan N, Yang DC, 2010. Molecular identification of oriental medicinal plant *Anemarrhena asphodeloides* Bunge ("Jimo") by multiplex PCR. *Mol Biol Rep* 37: 955-960.

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.

Lin J, Zhou X, Gao S, Wu W, Liu X, Sun X, Tang K, 2006. Authentication of *Pinellia ternata* and its adulterants based on PCR with specific primers. *Planta Med* 72: 844-847.

Meudt HM, Clarke AC, 2007. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends Plant Sci* 12: 106-117.

Mueller UG, Wolfenbarger LL, 1999. AFLP genotyping and fingerprinting. *Trends Ecol Evol* 14: 389-394.

Mukherjee PK, Pitchairajan V, Murugan V, Sivasankaran P, Khan Y, 2010. Strategies for revitalization of traditional medicine. *Chin*

Herb Med 2: 1-15.

Pang X, Song J, Zhu Y, Xie C, Chen S, 2010. Using DNA barcoding to identify species within Euphorbiaceae. *Planta Med* 76: 1784-1786.

Passinho-Soares H, Felix D, Kaplan MA, Margis-Pinheiro M, Margis R, 2006. Authentication of medicinal plant botanical identity by amplified fragmented length polymorphism dominant DNA marker: Inferences from the *Plectranthus* genus. *Planta Med* 72: 929-931.

Qian L, Ding G, Zhou Q, Feng Z, Ding X, Gu S, Wang Y, Li X, Chu B, 2008. Molecular authentication of *Dendrobium loddigesii* Rolfe by amplification refractory mutation system (ARMS). *Planta Med* 74: 470-473.

Qin J, Leung FC, Fung Y, Zhu D, Lin B, 2005. Rapid authentication of ginseng species using microchip electrophoresis with laser-induced fluorescence detection. *Anal Bioanal Chem* 381: 812-819.

Ruzicka J, Lukas B, Merza L, Göhler I, Abel G, Popp M, Novak J, 2009. Identification of *Verbena officinalis* based on ITS sequence analysis and RAPD-derived molecular markers. *Planta Med* 75: 1271-1276.

Sasaki Y, Komatsu K, Nagumo S, 2008. Rapid detection of *Panax ginseng* by loop-mediated isothermal amplification and its application to authentication of Ginseng. *Biol Pharm Bull* 31: 1806-1808.

Sasaki Y, Nagumo S, 2007. Rapid identification of *Curcuma longa* and *C. aromatica* by LAMP. *Biol Pharm Bull* 30: 2229-2230.

Sass C, Little DP, Stevenson DW, Specht CD, 2007. DNA barcoding in the cycadales: Testing the potential of proposed barcoding markers for species identification of cycads. *PLoS One* 2: e1154.

Seberg O, Petersen G, 2009. How many loci does it take to DNA barcode a crocus? *PLoS One* 4: e4598.

Sharma RK, Gupta P, Sharma V, Sood A, Mohapatra T, Ahuja PS, 2008. Evaluation of rice and sugarcane SSR markers for phylogenetic and genetic diversity analyses in bamboo. *Genome* 51: 91-103.

Shaw PC, Wang J, But PPH, 2002. Authentication of Chinese medicinal materials by DNA technology. Singapore: World Scientific.

Smillie TJ, Khan IA, 2010. A comprehensive approach to identifying and authenticating botanical products. *Clin Pharmacol Ther* 87: 175-186.

Song J, Yao H, Li Y, Li X, Lin Y, Liu C, Han J, Xie C, Chen S, 2009. Authentication of the family Polygonaceae in *Chinese Pharmacopoeia* by DNA barcoding technique. *J Ethnopharmacol* 124: 434-439.

Su H, Wang L, Liu L, Chi X, Zhang Y, 2008. Use of inter-simple sequence repeat markers to develop strain-specific SCAR markers for *Flammulina velutipes*. *J Appl Genet* 49: 233-235.

Sucher NJ, Carles MC, 2008. Genome-based approaches to the authentication of medicinal plants. *Planta Med* 74: 603-623.

Suo FM, Song JY, Chen SL, Wang YG, Mei HF, Eiji M, Osami T, Xie CX, 2010. AFLP analysis on genetic relationship among *Rheum tanguticum*, *Rheum palmatum*, and *Rheum officinale*. *Chin Tradit Herb Drugs* 41(2): 292-296.

Sze SC, Zhang KY, Shaw PC, But PP, Ng TB, Tong Y, 2008. A DNA microarray for differentiation of (Fengdou Shihu) by its 5 S ribosomal DNA intergenic spacer region. *Biotechnol Appl Biochem* 49: 149-154.

Tamhankar S, Ghate V, Raut A, Rajput B, 2009. Molecular profiling of "Chirayat" complex using inter simple sequence repeat (ISSR) markers. *Planta Med* 75: 1266-1270.

Techen N, Crockett SL, Khan IA, Scheffler BE, 2004. Authentication of medicinal plants using molecular biology techniques to compliment conventional methods. *Curr Med Chem* 11: 1391-1401.

Tsoi PY, Woo HS, Wong MS, Chen SL, Fong WF, Xiao PG, Yang MS, 2003. Genotyping and species identification of *Fritillaria* by DNA chips. *Acta Pharm Sin* 38: 185-190.

Wang C, Zhou TH, Yang X, Guo J, Zhao GF, 2008. Identification of seven plants of *Gynostemma* BL. by ISSR-PCR. *Chin Tradit Herb Drugs* 39(4): 588-591.

Wang ZA, Xu X, Shen XX, Chen BL, Shou HX, 2008. Establishment and application of AFLP fingerprinting system in Atractylodes macrocephala germplasm. *J Chin Med Mater* 31: 483-487.

Watthanachaiyingcharoen R, Komatsu K, Zhu S, Vajragupta O, Leelamanit W, 2010. Authentication of *Coscinium fenestratum* among the other menispermaceae plants prescribed in Thai folk medicines. *Biol Pharm Bull* 33: 91-94.

Xue CY, Li DZ, Wang QZ, 2009. Application of LightCycler polymerase chain reaction and melting curve analysis to the authentication of the traditional Chinese medicinal plant *Cimicifuga foetida*. *Planta Med* 75: 873-875.

Xue CY, Xue HG, 2008. Application of real-time scorpion PCR for authentication and quantification of the traditional Chinese medicinal plant *Drynaria fortunei*. *Planta Med* 74: 1416-1420.

Yao H, Song JY, Ma XY, Liu C, Li Y, Xu HX, Han JP, Duan LS, Chen SL, 2009. Identification of *Dendrobium* species by a candidate DNA barcode sequence: The chloroplast *psbA-trnH* intergenic region. *Planta Med* 75: 667-669.

Zhu S, Fushimi H, Komatsu K, 2008. Development of a DNA microarray for authentication of ginseng drugs based on 18S rRNA gene sequence. *J Agric Food Chem* 56: 3953-3959.