QUALITY CONTROL FOR CHINESE HERBAL DRUGS USING DNA PROBE TECHNOLOGY

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INTRODUCTION

The type and spectrum of diseases are changing significantly as the society aging today. The autoimmune diseases such as senile dementia , AIDS , as well as cardio-cerebral vascular diseases such as hypertension , arrhythmia , myocardiac infarction are becoming an intractable and globular problem. Recently , people are very much concerned with side effects of synthetic pharmaceuticals and are anxious to return to the use of natural medicine . Back to nature , the need for Chinese herbal drugs is increasing gradually for prevention and therapy of diseases in the world .

A recent survey in China has found about 10 000 species of medicinal plants and animals , and about 500 of them are frequently used. The accurate identification of these medicinal materials is a prerequisite for the quality control of Chinese herbal drugs . Traditional means of authentication rely on the inspection of organoleptic markers such as shape , color , texture and odor of the material . The accuracy of these methods depends heavily on the examiner's experience and , therefore , is prone to uncertainty as the criteria used are often subtle and ambiguous .

With the advance of modern technologies , anatomical and chemical analyses have been developed. Two common methods are thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) . Due to simplicity and low cost , TLC has become a popular means of revealing chemical constituents . The TLC profiles of several herbal drugs have now been recorded in the Chinese Pharmacopoeia (2000 edition) and Japanese Pharmacopoeia (13th edition) . Nevertheless , there are limitations in chemical and anatomical analyses . The markers are affected by physiological conditions , age , harvesting time and storage conditions . The chemical profiles of some medicinal materials may be too complicated and difficult to reproduce . Moreover , it is difficult to separate closely related species from one another as they contain similar chemical components . Recently , techniques with increased sensitivity and accuracy such as capillary electrophoresis (CE) as well as the combination of chromatography and mass spectrometry (e.g. , GC-MS , LC-MS-MS) have also been found to be good means in quality control of Chinese herbal drugs .

The use of soluble proteins and isozymes for authentication has also been reported in many medicinal materials . However , the informative markers for closely related strains are limited . In addition , the patterns of protein may vary in different tissues , developmental stages and environment as a result of temporal and spatial gene expression .

Advances in molecular biology have offered an additional tool for the authentication of medicinal materials . The use of DNA has several significant advantages : (1) genotype rather than phenotype are assayed ; (2) the methods are applicable to DNA from any source ; and (3) DNA can be prepared from a

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small amount of tissue. This is of particular importance as many medicinal samples available for examination are in limited supply.

DNA sequencing focuses on a defined region on the genome and produce well-defined conclusions, since different regions on the genome evolve to different extents, by choosing the target DNA carefully, organisms of different systematic levels can be compared.

Some Chinese herbal drugs, particularly crude animal drugs, have often been faked by the counterpart of other similar drugs. Since the early 1990s, DNA sequencing of different genes has been adopted for quality control of Chinese herbal drugs[1-5]. The cytochrome b (*cyt*-b) and 12S rRNA gene sequences of mitochondrial DNA (mt DNA) from animal herbal materials have been used for authentication purposes. The sequence of ribosomal RNA (5S, 18S, 25S rRNA), internal transcribed spacer (ITS) from nuclear ribosomal DNA (nrDNA), and transfer RNA for lysine (*trn*K) containing its open reading frame (ORF, called as *mat*K), rubisco large subunit (*rbc*L) from chloroplast DNA (cp DNA) is another locus for authentication of plant herbal materials. The rRNA and ITS typically consists of several hundred repeated copies of the transcription unit and non-transcribed spacer. Owing to concerted evolution, the various copies usually have homogeneous sequence and can be treated as a single unit, which is exactly to our wishes that make up drawback of degradation of DNA in dried herbal drugs.

The following examples describe the probe design principle, PCR condition optimization and detection of PCR product and to illustrate the application of DNA probe in authentication of Chinese herbal drugs based on the polymorphic site mutation information of DNA sequence of samples examined.

SPECIFIC DETECTION OF CHINESE HERBAL DRUGS

Specific primer marker

The design principle of species-specific primer is that the oligonucleotide of newly-designed probe must be exactly paired with DNA template of the target sample but be not completely paired with that of other samples, particularly in 3′-end nucleotide position of the primer. The PCR condition is high stringency amplification reaction with the primer specific to a given sample to distinguish other samples.

Because the primer is specifically designed based on a defined region of gene sequence, the amplification of PCR under highly stringent condition eliminated non-specific binding of primer to template DNA. The specific primer used results in a fingerprint that is much simpler to interpret as only one band is amplified. So, the time and cost for identification analysis of Chinese herbal drugs are greatly reduced.

We successfully explored to examine the origins of three *Panax* plants and their corresponding ginseng drugs using mutant allele specific amplification (MASA), i.e. amplification refractory mutation system (ARMS) method[6] (Figs 1, 2). 18S rRNA gene was amplified by PCR using a pairwise of consensus primers from the sequence flanking the nuclear ribosomal DNA small subunit region. As a result, for three *Panax* plants *P. ginseng*, *P. quinquefolius* and *P. japonicus*, PCR products with 1.8 kb in size were obtained. Subsequently, the nucleotide sequences of these PCR products were determined.

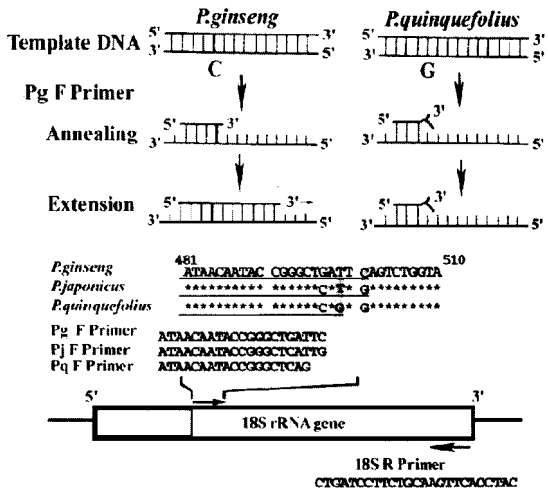

Fig 1 Rationale of ARMS technique and design of specific primer (Pg F, Pj F and Pq F) used for identifying *Panax* drugs

After sequence is aligned, three kinds of oligonucleotide forward primers, namely Pg F, Pq F and Pj F, were synthesized. Each primer has a sequence corresponding to each *Panax* plants at nucleotide positions 481 to 499 or 501 at the upstream of the 18S rRNA gene, where different base substitutions were observed.

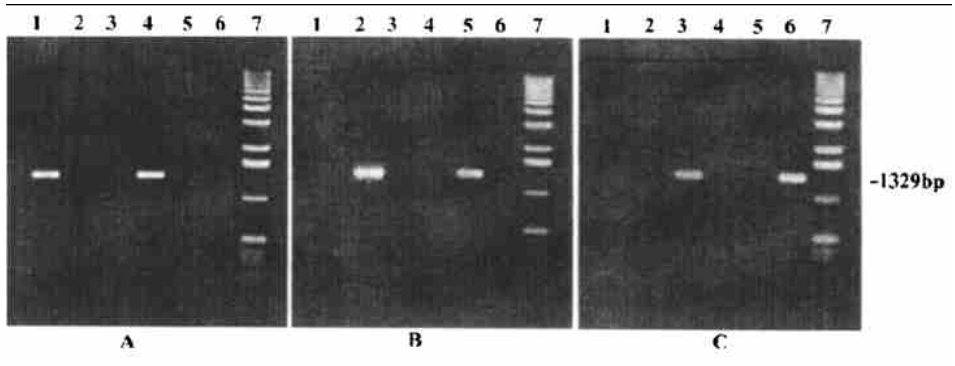

Fig 2 Result of ARMS analysis using specific primers Pg F (A) , Pj F (B) and Pq F (C)
Lane 1 : *Panax ginseng*; Lane 2 : *P. japonicus*; Lane 3 : *P. quinquefolius*; Lane 4 : Radix Ginseng; Lane 5 : Rhizoma Panacis Japonici; Lane 6 : Radix Panacis Quinquefolii; Lane 7 : 1 kb ladder

Specific restriction enzyme marker

a) Amplification fragment length polymorphism (AFLP)

AFLP is multi-locus approach based on endonuclease digestion of total genomic DNA. The basic steps are first endonuclease digestion of genomic DNA, then synthetic adapters are ligated to the restriction fragments using ligase, and selective PCR are performed. Finally the amplified fragments are resolved by denatured polyacrylamide gel electrophoresis. As a higher level of polymorphism can be recorded, the method is particularly suitable for assessment of intra-species relatedness. AFLP analysis has been used for species identification of two *Panax* plants such as *P. ginseng* and *P. quinquefolius*[7-10].

b) Polymerase chain reaction-selective restriction (PCR-SR)

In PCR-SR, a modified form of classic restriction fragment length polymorphism (RFLP), a defined DNA sequence is first amplified by PCR, and then selected restriction enzymes are used to cleave the amplified region to generate fragments unique to the species concerned.

PCR-SR analysis has been carried out on some *Atractylodes* plants and drugs according to ITS1 gene sequence from nuclear genome[11] and *trn*K gene sequence from chloroplast genome[12]. Our research group have performed the origin identification of a toxic herbal drug Banxia (*Pinellia ternata*) by 18S rRNA gene sequencing[13].

PCR-SR analysis was sought on the basis of each sequence of the 18S rRNA gene from four plants of Banxia and its three adulterants Huzhang (*Pinellia pedatisecta*), Tiannanxing (*Arisaema heterophyllum*) and Shuibanxia (*Typhonium flagelliforme*) by direct PCR sequencing technique. The restriction enzyme *Ase* I was found to give distinctive diagnostic fragments among Banxia and three other adulterants. After selection of a pairwise of universal primer set (18S F and 18S R) based on the whole region (nucleotide position 1 - 1805) of 18S rRNA sequence detected, the PCR is routinely performed under general conditions, the PCR product was digested with 10 units of restriction enzyme *Ase* I at 37 ℃ for 1 hour, fractionated by 3 % agarose gel electrophoresis. The restriction enzyme *Ase* I recognizes the sequence 5′-AT↓TAAT-3′, there is one recognition site of restriction enzyme *Ase* I in the 18S rRNA sequence of Banxia at the nucleotide position 967 - 972, that is not in one of its adulterations *P. pedatisecta*, *Arisaema heterophyllum* and *Typhonium flagelliforme* (Figs 3, 4). In the electrophoresis profile, the PCR product on the 18S rRNA gene of Banxia digested with *Ase* I showed two fragments of 837 bp and 968 bp, while in its three adulterants, the products showed an undigested fragment of 1805 bp. Thus, the use of restriction enzyme *Ase* I was suitable for PCR-SR identification between Banxia (*P. ternata*) and its adulterants Huzhang, Tiannanxing and Shuibanxia.

Other specific marker

a) Sequence characterized amplified region (SCAR)

The main steps of this method are first identification of a polymorphic band using RAPD, then nucleotide sequencing of the polymorphic band, and finally PCR reproduction of the polymorphic band as template DNA using a pair of long primer (25 - 30 mer) that are specific to the band. The conversion of

a RAPD to a SCAR improves reproducibility of PCR product and enhances the discriminatory power and reliability of the identification method. This method has been used for detecting Oriental ginseng and American ginseng[14].

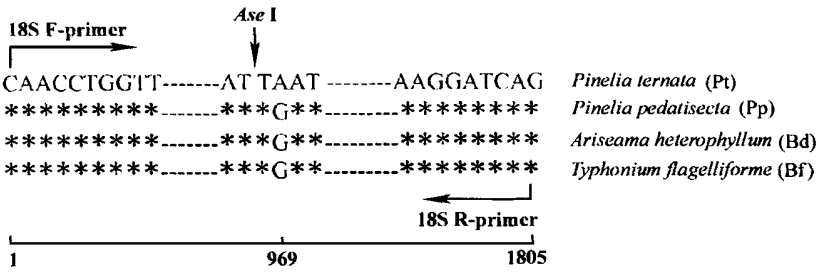

Fig 3 *Ase* I recognition site in the 18S rRNA gene region of *Pinellia ternata* and its three alias. The arrow represents direction of PCR amplification. The numbers below indicate the nucleotide position. Asterisks indicate nucleotide identity with *Pinellia ternata*

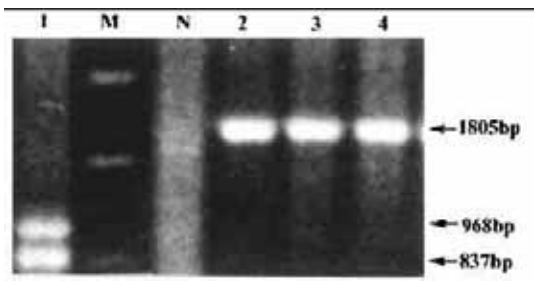

Fig 4 Agarose gel electrophoresis pattern of PCR-SR analysis from Banxia and its adulterants
Lane 1 : Pt ; Lane 2 : Pp ; Lane 3 : Bf ; Lane 4 : Bd ; Lane N: negative control without template DNA; Lane M: 1 kb ladder. The code of samples are indicated in Fig 3

b) Direct amplification of length polymorphism (DALP)

The DALP method uses an Arbitrary primer-PCR to produce genomic fingerprints, the oligonucleotides in use share the same forward 5′ core sequence but differ in 2 to 5 selective nucleotide at the 3′ end. Each primer is used in combination with a corresponding reverse primer and the PCR amplification is performed under high stringency. Such pair of primers can produce specific multi-banded patterns where length variation among individual samples can also be revealed. It is reported that some polymorphic DNA fragments of Oriental ginseng were produced using DALP, then cloned and sequenced, one of them is mini-satellite sequence unique to *Panax* ginseng which the specific sequence marker is used to design a PCR primer for rapid identification of ginseng[8].

QUANTITATIVE DETERMINATION OF CHINESE HERBAL DRUGS

RT-PCR and DD-based bioassay system

In the search for natural products exhibiting defined biological activity, it is essential to establish a bioassay system that can accurately evaluate the corresponding activity. Until now, a variety of bioassays using whole animals, isolated tissues and cell cultures have been developed and used for screening natural products and chemical compounds as well as prescriptions from Chinese herbal drugs. In addition, *in vitro* bioassays at protein level have been developed to search for specific modulators of important proteins such as enzymes and antiserum[15].

Since molecular cloning of the gene which code these important proteins is progressing rapidly, it makes the construction of new bioassay systems at the gene level possible. One feature of the new bioassay system is the application of quantitative reverse transcribed-PCR (RT-PCR) to determine changes in the transcription of a target gene using competitive internal standard DNA, because RT-PCR is a rapid and highly sensitive method for the detection of mRNA whose differential display (DD) is a technique detecting gene with very low expression levels. As the first application of the new bioassay strategy, the expression of the interleukin-2 (IL-2) gene in Jarkat cell (a human T cell line) and mouse testosterone 16-alpha-hydroxylase gene (cytochrome $P\text{-}450_{16\alpha}$) was investigated from several Chinese herbal drugs such as *Coptis chinensis*, *Pinellia ternata*, and Xiao-Chai-

Hu Tang[16,17]. Preliminary experiments show that RT-PCR-DD bioassay system may evaluate modulation of target gene expression after treatment of Chinese herbal drugs and their prescription such as Xiao-Chai-Hu Tang.

DNA chip (DNA microarray)-based identification system

Proceedings of DNA array technology opened the way for the high throughout gene analysis, such as sequence analysis by hybridization (SBH) or gene expression monitoring (GEM). SBH, according to the ability to determine a DNA sequence by hybridizing oligonucleotide probes to the target DNA, appears to be a method with great potential for megabase sequencing (Fig 5). This assay may lead to the discovery of novel mechanisms of action for newly identified or presently known compounds, as bioactive substances isolated through this bioassay system are predicted to have effects on some signal transduction involved in the control of target gene expression. They will be useful tools in the molecular mechanism of cellular signaling related to gene expression. Concerned experiments are progressing in some laboratories. We first developed a simple DNA chip for detecting two Chinese herbal drugs, Banxia (*Pinellia ternata*) and Huzhang (*P. pedatisecta*), based on the sequence from nuclear ribosomal RNA region[18] (Fig 6).

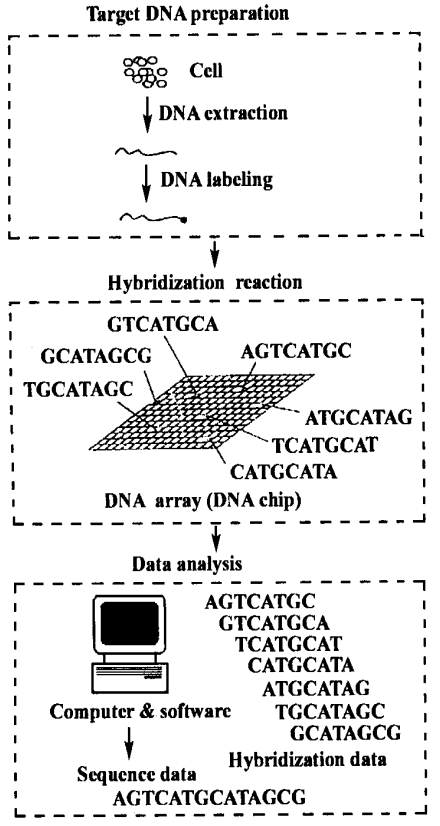

Fig 5 Brief rationale of sequence analysis by hybridization (SBH)

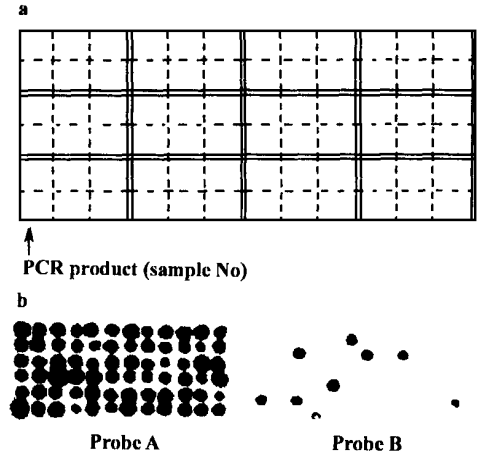

Fig 6 The preparation of DNA chip (a) and preliminary identification results of Banxia and Huzhang (b)

(a): Nylon filter was used as solid phase of DNA chip, its dot size is 6 mm × 3 mm (length × width). (b): Probe A (ATGTTTTCATTAATCAAG) is Banxia-specific which could be used for detecting all 72 samples of Banxia; Probe B (ATGTTTTCATTGATCAAG) is Huzhang-specific, for detecting only 9 samples of Huzhang from a total of 72 samples

CONCLUSIONS

Although PCR direct sequencing-based DNA probe method must first require the genetic background of the target genome, the reliability of these results cannot be greatly influenced by experimental conditions. DNA probe marker for the quality control of Chinese herbal drugs has several significant advantages over morphological and chemical markers for this purpose.

We, therefore, conclude that DNA probe technology has proven to be important in definitive authentication of species-origin of Chinese herbal drugs. To increase the practicability and reliability of quality control, it may be essential to combine DNA probe (large molecular bioassay) with chemical means (small molecular analysis) which looks mainly for the content in the bioactive components.

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DNA探针技术在中药材品质监控方面的研究与应用

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关键词: 中药材; DNA芯片; DNA探针; 品质监控