Review

Genetic authentication of ginseng and other traditional Chinese medicine

C C HON, Y C CHOW, F Y ZENG, F C C LEUNG[1]

Department of Zoology, The University of Hong Kong, Hong Kong SAR, China

KEY WORDS ginseng; saponins; microsatellite; random amplified polymorphic DNA technique; restriction fragment length polymorphism; amplified fragment length polymorphism; Chinese traditional medicine

ABSTRACT

The main objective of this paper is to review the chemical and genetic methods used in authentication of ginseng, especially the recent advances in microsatellite genotyping and its application to the authentication of other traditional Chinese medicines (TCM). The standardization and modernization of TCM hinge on the authentication of their botanical identities. Analysis of well-characterized marker compounds is now the most popular method for identifying the herbal materials and quality control of TCM, eg, ginsenoside profiling for authentication of *Panax* species. However, in many herbal species the chemical composition of the plant changes with the external environment and processing conditions, which lowers the reliability of these authentication methods. In the light of the advances in molecular biotechnology in the past few decades, genetic tools are now considered to provide more standardized and reliable methods for authentication of herbal materials at the DNA level. These genetic tools include random amplified polymorphic DNA (RAPD), DNA fingerprinting using multi-loci probes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and microsatellite marker technology. The practicality of these methods varies in terms of their sensitivity, reliability, reproducibility, and running cost. Using ginseng as an example, we reviewed the advantages and limitations of these molecular techniques in TCM authentication. We have developed a set of microsatellite markers from American ginseng that are able to differentiate *Panax ginseng* and *Panax quinquetolius* with the resolution down to farm level, ie, confirmation of its botanical identity and origin. Compared with other molecular techniques, microsatellite marker technology is more robust, accurate, reproducible, reliable, and sensitive. This is essential for large-scale TCM authentication centers.

INTRODUCTION

The therapeutic value of traditional Chinese medicines (TCM), especially on chronic diseases, has won wide acknowledgement in the West in recent decades. The TCM seems to make enormous strides following China's entry into the World Trade Organization (WTO), as large sums of capital investment will now become available to spur technical innovation. Many world-famous medicine companies including Pfizer and Johnson & Johnson have shown great interest in developing TCM jointly with Chinese companies. The World Health Organization (WHO) has also been keen to pursue the development of TCM in recent years. Authen-

[1] Correspondence to Prof FCC LEUNG.
E-mail fcleung@hkucc.hku.hk
Received 2003-02-18 Accepted 2003-04-18

tication and quality control have been the key for a Chinese herbal drug to enter the world market. Unlike most western pharmaceuticals, TCM are usually the herbal plants themselves, or formulations of the herbal plants or extracts consisting of thousands of biological compounds rather than a single or simple combination of several chemicals. Thus its chemical complexity makes the quality control process much more sophisticated, and current pharmaceutical Good Manufacturing Practices (cGMP) cannot be applied to such herbal products. Traditionally, histological and morphological inspections have been the usual methods of authentication, but they are not applicable to most forms of modern herbal drugs, eg, herbal extracts and pills. Chemical and chromatographic techniques are currently used for identification and assessment of chemical constituents of TCM. The most common techniques are high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and gas chromatography (GC)[1]. Chromatographic fingerprints and marker compounds are used as reference standards, which indicate the purity, identity, and quality of the herbal drug. However, chemical variability within the plant materials hinders the confirmation of its botanical identity. The chemical composition of a herbal species varies from grower to grower and crop to crop, and is significantly affected by growth and storage conditions and harvest and post-harvest processes. Genetic makeup inspections provide a definite answer to the botanical identity of the TCM, as the genetic makeup of a herbal species does not vary with their physical form, physiological and external conditions. With the advances in molecular biotechnology in the past decades, different DNA manipulation techniques have been developed and can potentially be used in TCM authentication. Using *Panax* species as an example, the methods developed previously include low-Cot DNA fingerprinting[2,3], randomly amplified polymorphic DNA (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR)[4-6], PCR-restriction fragment length polymorphism (PCR-RFLP) and MASA analysis[7], amplified DNA RFLP[8] and microsatellite markers (unpublished data). This paper reviews the application of these technologies in authentication of *Panax* species, with particular reference to microsatellite marker technology developed in our laboratory.

CHEMICAL AUTHENTICATION OF GINSENG

The chemical profile of ginsenosides has been employed for authentication of ginseng. Ginsenosides belong to a group of related phytochemicals called saponins, which are also found in many other plants. *Panax quinquefolius* has 29 different ginsenosides, a higher total percentage than the 20 ginsenosides found in *Panax ginseng* (British Columbia Ministry of Agriculture and Food, 1999). Ginsenoside R_f is Chinese ginseng specific while 24(*R*)-pseudoginsenoside F_{11} is American ginseng specific, and they have been chosen as the indicative ginsenosides for species differentiation[9]. Different analytic approaches have been developed and HPLC in conjunction with mass spectrometry (MS) developed by Chan *et al*[9] appears to be the most promising. Chan *et al*[9] proved more sensitive and specific methods than commonly used TLC methods. Besides the indicative ginsenoside R_f and F_{11}, they also showed that the ginsenoside ratios Rg_1/Re and Rb_2/Rc were specific for these two species of ginseng. They found some brands of ginseng slice and tea granules did not contain the labeled constituents described by the manufacturers. Moreover, origins of ginsengs can also be indicated by the relative peak heights of the indicative ginsenoside markers, but the resolution is very limited[9]. This method is sensitive and robust. However, as mentioned before, quantitative variations in ginsenosides between samples within the same *Panax* species do not give us complete confidence in the confirmation of the botanical identity of the drug.

GENETIC AUTHENTICATION OF GINSENG

Genetic differences between *Panax* species provide a more discrete, standard, and definite means of botanical identification than chemical methods. Moreover, genetic differences are always more informative than phenotypic differences such as morphology and chemical compositions, and can be used to authenticate not only identity but also origin. Many molecular techniques have been developed for studying the genetic diversity of different organisms. Some of these techniques have been successfully employed in forensic science, such as DNA fingerprinting and microsatellite marker technology[10-12]. The success in applying these techniques in ecology and forensics science has encouraged the application of these genetic tools in TCM authentication. In the following section, the development of different genetic tools on ginseng authentication is discussed.

DNA fingerprinting using low-cot DNA probes

Jeffreys *et al*[11] introduced the concept of using large number of hypervariable DNA loci as DNA probe to generate an individual-specific DNA profile, which provides a powerful application to human forensics. The quality and resolution of a DNA fingerprinting assay much depended on the choice of the DNA probe. Leung *et al*[13] first developed a low-cot (rapid reassociating fraction) DNA probe method for the identification of host-specific DNA fragments in *Fusarium oxysporum* and it was found to be an excellent DNA probe for DNA fingerprinting. Leung and Ho[2,3] employed the same approach for DNA fingerprinting assay of ginseng, which generated a distinctive banding pattern with similarity index 0.55 between the two *Panax* species (Fig 1). The high similarity index agrees with the low internal transcribed spaces sequence divergence reported by Wen and Zimmer[14]. Although the fingerprints can differentiate Chinese and American ginseng, they cannot do so with sufficient resolution to permit accurate identification of their species and origin. Moreover, the stability and reproducibility of this method are very low because the quality of the assay depends on the quality of the DNA and technical factors, such as blotting and hybridization conditions. The major limitation of this method is its sensitivity, as it needs a large amount of high quality genomic DNA. Most genomic DNA molecules in the processed herbal drugs are degraded and unsuitable for DNA fingerprinting. Fingerprinting is laborious and incompatible with high analytical throughput applications.

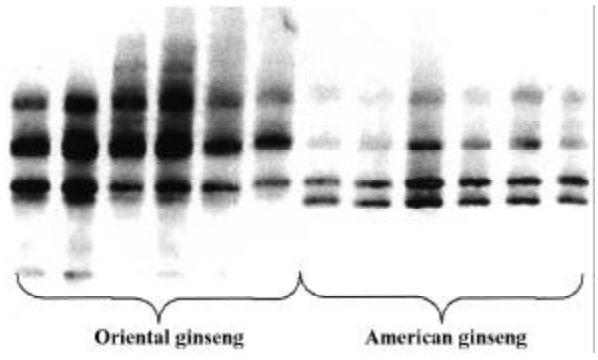

Fig 1. DNA fingerprints using low-Cot probes[3]. The fingerprint pattern differentiates two ginseng species with a low resolution.

Random-primed polymerase chain reaction RAPD refers to DNA fragments amplified by a polymerase chain reaction (PCR) using a single arbitrary primer[15,16], resulting in genetic polymorphisms observed between different genomes. This technique has frequently been used for the detection of genetic variability in plants. The advantages of the approach are its rapidity, simplicity, and the absence of any need for prior genetic information of the plant[17]. Cheung *et al*[17] differentiated American and Chinese ginseng roots by using AP-PCR. Shaw and But[6] further extended this technique on the authentication of *Panax* species and their adulterant. They obtained distinct RAPD fingerprints for American and Chinese ginseng, which are consistent irrespective of plant source or age. These distinct fingerprints of the two ginseng species showed a very different pattern from that of their adulterant. Bai *et al*[4] applied this method to the study of the genetic diversity of Ontario American ginseng. With 36 decamers, Bai *et al* tested through 48 ginseng plants, and achieved a mean similarity coefficient of 0.412. The limitation of this method is its reproducibility and relatively low resolution.

PCR-restriction fragment length polymorphism Ngan *et al*[8] and Fushimi *et al*[7] applied PCR-RFLP on ginseng authentication based on ribosomal ITS1-5.8S-ITS2 region and 18S rRNA gene respectively. Both studies showed promising results on identification of the two *Panax* species and their adulterants. Ngan *et al* are currently expanding this method (ITS1-5.8S-ITS2 region based) to authentication of many other TCM. This assay method is more reproducible and reliable than DNA fingerprinting and RAPD. However, the degree of polymorphism among individuals within a species is so limited that it is impossible to determine the origin of the plant.

Polymorphic microsatellite marker technology Microsatellites are very short tandem repeats (2-6 bp), which are usually highly polymorphic and informative. A unique fingerprint of an individual can be obtained by PCR amplification of these marker loci. This method has been used extensively in genetic and evolution studies or forensic sciences, for determining gender[18,19], settling questions of relatedness and parentage[20-23], determining the genetic structure of populations[24-27], and making genetic comparisons among species[28,29]. Human identification kits have been developed by Applied Biosystems based on discriminating fluorescent PCR microsatellite-based assay, which simultaneously amplifies 15 STR loci plus the Amelogenin gender-determining marker in a single, robust PCR. Several technical and analytical advantages make this technique superior to other genetic markers including its highly infor-

mative nature, accuracy, sensitivity, reliability, and reproducibility. This technique is robust, and can be fully automated and fitted into large-scale, high throughput authentication centers. The main limitation of microsatellite marker analysis is the high input in terms of cost and labor required for identification and screening of informative loci.

Microsatellite marker technology has not yet been introduced into authentication of TCM and our research team pioneered the use of this technology for the authentication of ginseng. We screened 16 microsatellite makers from American ginseng genomic library. Over 150 American ginseng roots and 40 Oriental ginseng roots were analyzed. Using 9 of the 16 screened loci, Chinese samples could be differentiated unambiguously from the American samples with an average difference of 1.7 in Nei's original genetic distance[30]. This degree of accuracy is sufficient to distinguish a ginseng species from a pool of samples of different species (Fig 2). High-resolution microsatellite markers can also be used to identify closely related genotypes of cultivars within a species. In a study on soybean performed by Rongwen *et al*[31], most of the 96 different genotypes investigated could be identified by a set of 7 informative loci exhibiting 11-26 alleles per locus. In our ownstudy, ten loci exhibited 4-11 alleles per locus, providing sufficient information for the ginseng species to be identified. Some of the informative loci showed quite different allelic patterns among different ginseng farms, implying that the allelic profile of an individual may reflect its origin (Fig 3).

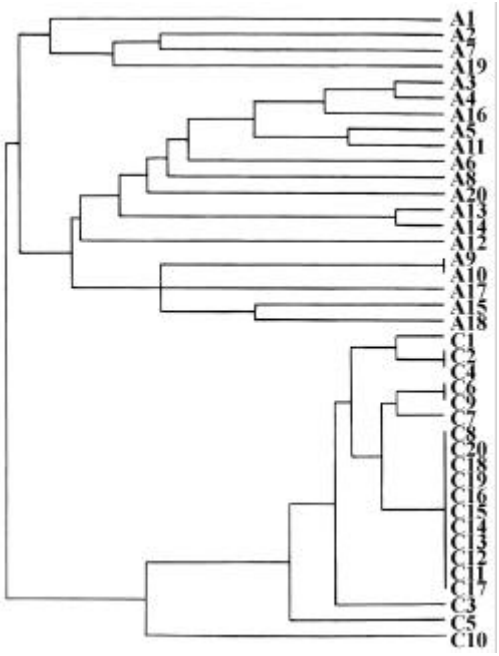

Fig 2. Dendrogram of 40 ginseng samples based on microsatellite genotyping. The dendrogram was constructed from the digitized microsatellite genotypes of the samples, and it differentiates Chinese and American ginseng samples unambiguously.

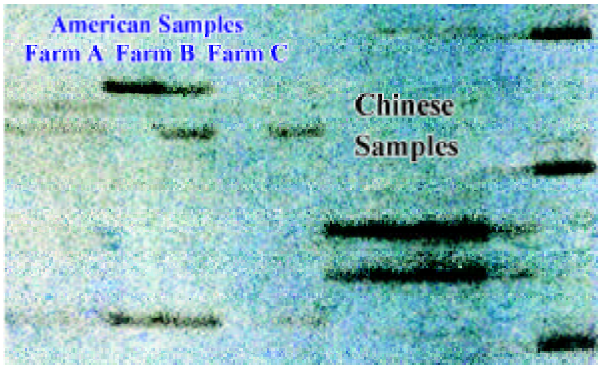

Fig 3. Genetic diversity with a ginseng species. The figure shows the allelic profile of a microsatellite locus in two ginseng species on a silver-stained PAGE. Polymorphism was detected on the farm level with American ginseng.

The allele-calling step is the bottleneck of the high throughput microsatellite analysis, as the allele size differences are usually less than 10 bp. In our preliminary analysis, we used silver stained PAGE for fragment analysis, which is labor intensive, time consuming and inaccurate. After identifying the informative loci, we turned to use ABI 310 genetic analyzer and GeneScan software for more efficient allele calling (Fig 4). However, it is still only "semi-automated."

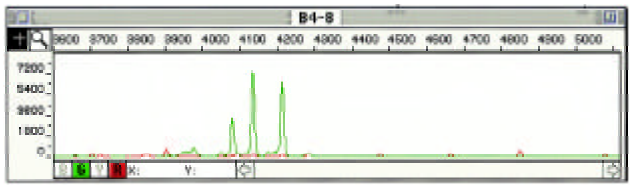

Fig 4. Genescan for allele calling. An electrogram of a locus in a ginseng sample generated from ABI Genetic Analyzer 310 and Genescan software. The peak location represents the accurate allele size and greatly reduced artificial error in the allele calling process.

High throughput and informative microsatellite analysis relies upon the following key factors: (i) polymorphic loci; (ii) advanced multiplex PCR technology; (iii) advanced capillary electrophoresis technology; (iv)

supporting robotics; and (v) automated allele calling and genotyping software. With today's technology, one single technician with adequate robotics could realistically produce 2880 genotypes per day, given a multiplex of 10 markers, 384-well PCR trays, 96-lane gels and a fully automated genotyping software (TrueAllele, Genotyper, GenoPedigree, etc). Such technology enables accurate and digitalized genotypes of herbal plant individuals to be obtained automatically with minimized artifacts and errors. The recent development of DNA array on microchip and the ability to read single base pair changes has expanded the concept of polymorphic DNA marker. However, such technology is still not mature enough to be applied to the authentication of TCM. Its potential for high throughput and automation makes microsatellite-based genotyping an attractive method for TCM authentication.

CONCLUSION

A standard and effective quality assurance program for TCM is necessary for their standardization, modernization, and acceptance by the world market. Chemical analysis using HPLC and TLC methods provides quantitative and qualitative insights into the chemical compositions, purity and quality of the drug. The authentication of the botanical identity of the herbal materials has to be standardized by genetic analysis on DNA level. Using ginseng as an example, various genetic tools have been employed in its authentication. Microsatellite marker technology was identified as the most suitable technique because of its resolution and efficiency. This technology provides discrete genotypes of a herbal plant, which permits its botanical identity and origin to be established beyond doubt. Nowadays the quality control of TCM focuses on different chemical analysis, and confirmation of the genetic identities of the herbs must be included in the quality assurance program in order to provide full confidence to consumers. The key requirement must be fulfill for TCM to enter the international market successfully.

REFERENCES

1 Natalie JL, Peter P. Use of fingerprinting and marker compounds for identification and standardization of botanical drugs: strategies for applying pharmaceutical HPLC analysis to herbal products. Drug Info J 1998; 32: 497-512.

2 Ho ISH, Leung FC. Isolation and characterization of repetitive DNA sequences from *Panax ginsen*g. Mol Genet Genomics 2002; 266: 951-61.

3 Leung FCC, Ho ISH. Development of low-cot DNA probe for fingerprinting Asia and North American ginseng. On-line Proceedings of the 5th Internet World Congress on Biomedical Sciences '98 at McMaster University. Canada; 1998.

4 Bai DP, Brandle J, Reeleder R. Genetic diversity in North American ginseng (*Panax quinquefolius* L.) grown in Ontario detected by RAPD analysis. Genome 1997; 40: 111-5.

5 Cheung KS, Kwan HS, But PPH, Shaw PC. Pharmacognostical identification of American and Oriental ginseng roots by genomic fingerprinting using arbitrarily primed polymerase chain reaction (AC-PCR). J Ethnopharmacol 1994; 42: 67-9.

6 Shaw PC, But PPH. Authentication of *Panax* species and their adulterants by random-primed polymerase chain reaction. Planta Med 1995; 61: 466-9.

7 Fushimi H, Komatsu K, Isobe M, Namba T. Application of PCR-RFLP and MASA analyses on 18S ribosomal RNA gene sequence for the identification of three ginseng drugs. Biol Pharm Bull 1997; 20: 765-9.

8 Ngan F, Shaw P, But PPH, Wang J. Molecular authentication of *Panax* species. Phytochemistry 1999; 50: 787-91.

9 Chan TW, But PPH, Cheng SW, Kwok IM, Law FW, Xu HX. Differentiation and authentication of *Panax ginseng*, *Panax quinquefolius* and ginseng products by the use of HPLC/MS. Anal Chem 2000; 72: 1281-7.

10 Gill P, Jeffreys AJ, Werett DJ. Forensic application of DNA "fingerprints". Nature 1985; 318: 577-9.

11 Jeffreys AJ, Brookfield JF, Semenoff R. Positive identification of an immigration test-case using human DNA fingerprints. Nature 1985; 317: 818-9.

12 Jeffreys AJ, Wilson V, Thein SL. Hypervariable "minisatellite" regions in human DNA. Nature 1985; 314: 67-73. Jeffreys AJ, Wilson V, Thein SL. Individual-specific "fingerprints" of human DNA. Nature 1985; 316: 76-9.

13 Leung FCC. New application of low-Cot DNA as a DNA fingerprint probe. Electrophoresis 1999; 20: 1762-7.

14 Wen J, Zimmer EA. Phylogeny and biogeography of *Panax* L (the Ginseng Genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. Am J Bot 1995; 85: 866.

15 Welsh J, McClelland M. Fingerprinting genomes by the use of PCR with arbitrary primers. Nucleic Acids Res 1990; 18: 7213-8.

16 Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990; 18: 6531- 5.

17 Cheng KT, Chang HC, Su CH, Hsu FL. Identification of dried rhizomes of *Coptis* species by the use of random amplified polymorphic DNA Bot. Bull Acad Sin 1997; 38: 241-4.

18 Delehanty DJ. Genetic and morphological methods for gender identification of mountain quail. J Wildl Man 1995; 59: 785-9.

19 Longmire JL, Maltbie M, Pavelka RW, Smith LM, White SM, Ryder OA. Gender identification in birds by the use of microsatellite DNA fingerprint analysis. Auk 1993; 110: 378-81.

20 Amos B, Schlötterer C, Tautz D. Social structure of pilot whales revealed by analytical DNA profiling. Science 1993; 260: 670-2.

21 Kellogg KA, Markert JA, Stauffer JR, Kocher TD. Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi. Afri Proc Royal Soc (London B) 1995; 260: 79-84.
22 McDonald DB, Potts WK. Cooperative display and relatedness among males in a lek-mating bird. Science 1994; 266: 1030-2.
23 Primmer CR, Moller AP, Ellegren H. Resolving genetic relationships with microsatellite markers: a parentage testing system for the swallow *Hirundo rustica*. Mol Ecol 1995; 4: 493-8.
24 Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR, Cavalli-Sforza LL. High resolution of human evolutionary trees with polymorphic microsatellites. Nature 1994; 368: 455-7.
25 Dallas JF, Dod B, Boursot P, Prager EM, Bonhomme F. Population subdivision and gene flow in Danish house mice. Mol Ecol 1995; 4: 311-20.
26 Estoup A, Garnery L, Solignac M, Cornuet JM. Microsatellite variation in honey bee (Apis mellifera L) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 1995; 140: 679-95.
27 Taylor AC, Sherwin WB, Wayne RK. Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. Mol Ecol 1994; 4: 277-90.
28 Roy MS, Geffen ED, Smith EA, Ostrander, Wayne RK. Patterns of differentiation and hybridization in North American wolflike canids, revealed by analysis of microsatellite loci. Mol Biol Evol 1994; 11: 553-70.
29 Garza JC, Slatkin M, Freimer NB. Microsatellite allele frequencies in humans and chimpanzees, with implications for constraints on allele size. Mol Biol Evol 1995; 12: 594-603.
30 Nei M. Genetic distances between populations. Am Nature 1992; 106: 83-92.
31 Rongwen J, Akkaya MS, Bhagwat AA, Lavi U, Cregan PB. The use of microsatellite DNA markers for soybean genotype identification. Theor Appl Genet 1995; 90: 43-8.