

Chapter 1

Molecular Markers in Chinese Medicinal Materials

Pang-Chui Shaw, Fai-Ngor Ngan, Paul Pui-Hay But and Jun Wang

Introduction

Traditional Chinese medicine (TCM) has been used for millennia in China. With its multi-target effects, TCM has been recognised as being particularly suitable for treating modern diseases such as cardiovascular diseases, asthma and other long-term illnesses. Furthermore, an increasing variety of health-care products have been developed from TCM to meet the contemporary trend of ‘back to nature’. At present, over 10,000 species of plants, animals and minerals have been found to have medicinal value and about 10% are frequently used in TCM. Substitutes and adulterants of TCM materials are often introduced intentionally or accidentally, thus seriously interfering with their therapeutic effects, even leading to life-threatening poisoning. In 1989, two people in Hong Kong suffered serious neuropathy and encephalopathy after consuming a broth from the roots of *Podophyllum hexandrum* (guijiu), a toxic herb disguised as *Gentiana rigescens* (longdancao). The same herb was also found as a contaminant in samples of *Clematis* roots (weilingxian), leading to fourteen reported cases of neuropathy in Hong Kong (But *et al.*, 1996). Four cases of drowsiness were attributed to erroneous dispensing of the anticholinergic Yangjinhua (the flower of *Datura metel*), instead of Lingxiaohua (the flower of *Campsis grandiflora*) (But, 1994). Adulteration resulting in an epidemic of severe kidney damage was reported in Belgium (Vanherweghem *et al.*, 1993; Vanhaelen *et al.*, 1994), where the herb *Stephania tetrandra* (hanfangji) was substituted with the nephrotoxic *Aristolochia fangchi* (guangfangji).

1.1. Existing Tools in the Authentication of TCM

The absence of an objective and accurate inspection system is frequently cited as one of the major reasons for herbal poisoning. Traditional means for authentication mainly depend on morphological and histological characteristics. Morphological identification includes the inspection of organoleptic markers such as shape, colour, texture and odour of the herbs. This method is simple and direct but its accuracy depends heavily on the examiners' experience and judgement, which is subjective and error-prone. It is also difficult to identify processed herbal materials when they are in powdered form or in shredded pieces. A general comparison of different authentication methods is shown in Table 1.

Table 1. Comparison of technical skill level and cost required for various authentication methods

| Authentication Methods | Skill | Cost |
|----------------------------|-------|------|
| 1. Organoleptic Inspection | +++ | + |
| 2. Protein Analysis | +++ | +++ |
| 3. TLC | ++ | ++ |
| 4. HPLC/CE/MS | +++++ | ++++ |
| 5. DNA Techniques | +++ | +++ |

'+++++' indicates the highest degree, '+' indicates the least

1.1.1. Anatomical Analysis

Microscopic examination is frequently used to reveal the characteristic cell components or tissues of a medicinal material or its processed by-product. For example, in the differentiation of *Panax ginseng* from *Phytolacca acinosa*, the internal structure of the ginseng root follows the pattern of the root in most dicotyledon plants. From the outside inwards it contains a protective periderm layer (skin), phloem, cambium, xylem with vessels, fibres and the central pith. However, *P. ginseng*, but not *Phytolacca acinosa*, has resin canals around the cambium layer, an irregular gap in primary

phloem and rays bearing crystals of calcium oxalate (Hu, 1976; Kubo *et al.*, 1980; Tani *et al.*, 1980). Anatomical analysis may not provide an unequivocal means of authentication as different medicinal materials, particularly from related species, often have similar characteristics. Moreover, other factors including geographical environment, growth period and the storage condition of the herbs may affect the fine structure of the material.

1.1.2. Chemical Analysis

Chemical constituents are used as identification markers as well. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have become standard procedures in the identification of herbal materials. For example, in the HPLC analysis of *Panax* species, several ginsenosides have been used as markers. R_f is found only in *P. ginseng*, and R₁, but not R₀, is found in *P. notoginseng*. *P. quinquefolius*, however, has a high amount of R_{b1}. HPLC analysis has been applied to test the seventeen brands of American ginseng tea and seven brands of ginseng tea available in Hong Kong (Lang *et al.*, 1993). Recently, capillary electrophoresis has been used to infer botanical sources and to assess the quality of ephedrae herba (Liu *et al.*, 1993), coptidis rhizoma (Liu *et al.*, 1994), ginseng radix (Chuang *et al.*, 1995) and paeoniae radix (Lin *et al.*, 1996). Increased sensitivity has been achieved by coupling HPLC or gas chromatography with other analytical systems such as mass spectrometry. These include field desorption ionisation (FD) mass spectrometry (Schulten, 1982), 252Cf-plasma desorption (Elkin *et al.*, 1993) and liquid chromatography/mass spectrometry (LC/MS) (Wang *et al.*, 1999b; Chan *et al.*, 2000). Using the HPLC/MS method, it was possible to separate ginsenoside R_f and 24(r)-pseudoginsenoside f-11. The former is found in *P. ginseng* and the latter in *P. quinquefolius*. The two compounds were found to have similar retention times under most LC conditions (Chan *et al.*, 2000).

Nevertheless, there are limitations to chemical analysis. Contents of the active components may be affected by physiological conditions, harvesting period and storage conditions. Moreover, it is difficult to distinguish closely

related species, many of which contain similar chemical components. In addition, instruments such as HPLC, capillary electrophoresis and mass spectrometry are expensive and may not be available in many analytical laboratories.

1.1.3. Allozymes, Isozymes and Antibodies

The term 'allozyme' refers to the different allelic form of enzymes encoded by a one-locus gene whereas an 'isozyme' is encoded by alleles at more than one locus. In common practice, the latter refers to both classes. Since the 1960's, isozyme data have been used for characterising and identifying genotypes and varieties of crop plants and animals, for studying population genetics, for examining geographical patterns of variation and for linkage mapping. This technique has been used in identifying TCM materials as well. For examples: *Epimedium diphylum* is differentiated from other species by isozymes of isocitrate dehydrogenase, glutamate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase (Koga *et al.*, 1991); *Panax quinquefolius* and *P. ginseng* can be differentiated by electrophoresis of peroxidase, esterase, 6-phosphogluconate dehydrogenase, uridine-diphosphate glucose-pyrophosphorylase and glucose-phosphate isomerase (Zhuravlev *et al.*, 1999; Sun *et al.*, 1993) and *Ganoderma sinensis* has been shown to have a distinct relation with other strains by esterase isozyme analyses (Lan *et al.*, 1998).

However, on the average only 30% of the base changes in DNA lead to changes in amino acids, yielding a difference in net charges and thereby changing the electrophoretic mobility of the enzyme (Crawford, 1990). This means that there is a definite limit to the isozyme polymorphism. Hence, it is of no surprise that some species, even with apparent genetic variation in morphological traits, are monomorphic for most isozymes (Parker *et al.*, 1998). A further limitation, is that the level of isozymes is affected by the temporal and spatial regulation of gene expression and by environmental factors. It is evident in *Ganoderma* that the isozyme profile is variable, depending upon the stage of plant development (Lan *et al.*, 1998). Moreover, protein is prone to degradation after harvesting and prolonged storage.

An antibody is a very powerful authentication tool. It can distinguish isozymes as well as other proteins. A single difference in the amino acids may change the epitope of the protein which is recognised by the antibody. Accordingly, antibodies can differentiate polymorphic proteins and species-specific proteins from different organisms. For examples: antibodies were developed against *Prorocentrum lima* (Ehrenberg) Dodge and *P. rostratum* Stein (Lopez-Rodas and Costas, 1999); monoclonal antibodies were raised against a conjugate of ginsenoside Rf and bovine serum albumin; the anti-Rf monoclonal antibody was used for quantification of Rf in crude ginseng fractions and in body fluids (Nah *et al.*, 2000); an antibody specific to lumbricus (the crude drug dried earthworm) used in Chinese medicine was shown not to cross-react with other crude drugs (Bai *et al.*, 1997) and a selected antibody enzyme immunoassay was subsequently developed to measure the contents of the lumbricus component in herbal products.

Immunological analyses are highly reproducible and the antibodies may be developed into a diagnostic kit for rapid testing. However, tremendous effort is required for screening a suitable antigen and for generating the antibody.

1.2. Molecular Marker Technologies

Benefiting from molecular cloning and PCR techniques, DNA markers have now become a popular means for identification and authentication of plant and animal species. DNA-based markers are less affected by age, physiological condition of samples and environmental factors. They are not tissue-specific and thus can be detected at any phase of organism development. Only a small amount of sample is sufficient for analysis and the physical form of the sample does not restrict detection. These non-stringent requirements are particularly relevant for Chinese medicinal materials that are expensive or in limited supply. The power of discrimination of DNA-based markers is so high that very closely related varieties can be differentiated. In the following, we provide a general account of representative molecular marker technologies, which will be elaborated in the following chapters. Although RNA-based markers have also been considered, for simplicity, herein we shall focus primarily on DNA-based