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Molecular authentication of *Panax* species

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Abstract

Using conserved plant sequences as primers, the DNA sequences in the ribosomal ITS1–5.8S–ITS2 region have been amplified and determined for six *Panax* species, *P. ginseng* C. A. Mey. (Oriental ginseng), *P. quinquefolius* L. (American ginseng), *P. notoginseng* (Burkill) F. H. Chen (Sanchi), *P. japonicus* C. A. Mey. (Japanese ginseng), *P. trifolius* L. and *P. major* Ting, as well as two common adulterants of ginseng, *Mirabilis jalapa* L. and *Phytolacca acinosa* Roxb. An authentication procedure based upon the restriction fragment length polymorphism (RFLP) in the region is able to differentiate between *P. ginseng* and *P. quinquefolius*, and to discriminate the ginsengs from the two common poisonous adulterants. Broader application of this approach to authenticate other morphologically similar Chinese medicinal materials is rationalised.

Keywords: *Panax ginseng*; *P. quinquefolius*; Araliaceae; Ginseng; Traditional Chinese medicinal materials; Polymerase chain reaction; Authentication; Restriction fragment length polymorphism; Ribosomal DNA

1. Introduction

The genus *Panax* contains several medicinal species highly treasured in the Orient. The dried roots of *P. ginseng* C. A. Mey. (Oriental ginseng) and *P. quinquefolius* L. (American ginseng) have been applied as tonic, prophylactic and anti-ageing agents (Chang & But, 1986). The former is used in yang-deficient condition while the latter in ying-deficient condition. Dried roots of *P. notoginseng* (Burkill) F.H. Chen (Sanchi) are used as a hematostatic for haemorrhage and that of *P. japonicus* C.A. Mey. (Japanese ginseng) are used to smooth coughs and reduce phlegm (Chang & But, 1986). Other *Panax* species including *P. major* Ting are also used as medicines in many Asian countries including China, Japan, Korea and Vietnam. The ginseng trade is a big business; in 1993 Hong Kong alone imported 3.4 million kg of ginseng worth US$200 million (But, Li, & But, 1995). The illegal practice of disguising Oriental ginseng as American ginseng has become a common problem in recent years as the cultivated American ginseng commands a much higher market value than that of cultivated Oriental ginseng. Tremendous financial incentive also prompts dishonest merchants to imitate or adulterate ginsengs with certain herbal products including several poisonous plants that are morphologically similar to ginsengs. Two of such ginseng adulterants are *Mirabilis jalapa* L. and *Phytolacca acinosa* Roxb.

Obviously an effective program of authentication of Chinese herbs including ginsengs is essential to the healthy development of the herbal industry. It protects the commercial interests of consumers, minimises unfair competition among merchants, lowers the risk of misusing medicines and prevents the health hazard of many adulterants. Traditionally the authentication of Chinese herbs relies upon morphological and histological inspection. In many cases, as in the authentication of different *Panax* species, such an approach is far from reliable. The roots of *P. ginseng* and *P. quinquefolius* are very similar in morphological appearance. Furthermore many commercial ginseng products are in the form of powder or shredded slice, rendering their authentication by morphological and histological methods very difficult, if not impossible. Methods have been developed to authenticate ginseng samples by examination of their ginsenoside profiles (Yip, Lau, But, & Kong, 1985, 1985; Lang, Lou, & But, 1993). However, the application of chemical analysis may be limited as the amount and the profiles of ginsenosides are affected significantly by growth conditions as well as many other variables such as the storage condition, the freshness of the samples and the different post-harvest processing. In addition, the chemical

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method demands a large quantity of material for proper analysis.

Recently, DNA fingerprinting profiles characteristic of *P. ginseng* and *P. quinquefolius* have been generated from AP-PCR and RAPD (Cheung, Kwan, But, & Shaw, 1994; Shaw & But, 1995). Here we report a more reproducible and robust approach to authenticate each of the six *Panax* species and differentiate them from one another and from some of their adulterants.

2. Results and discussion

The DNA sequences in the region of ITS1–5.8S–ITS2 of rDNA were PCR-amplified from the roots of six *Panax* species *P. ginseng*, *P. quinquefolius*, *P. notoginseng*, *P. japonicus*, *P. trifolius* and *P. major*, and from the roots of two common adulterants of ginseng, *M. jalapa* and *Ph. acinosa*. The primers 18d and 28cc used for the amplification of ITS1 and ITS2 were complementary to the conserved regions of plant 18S and 26S rDNA (Hillis & Dixon, 1991) and, together with primers used in DNA sequencing, are indicated in Fig. 1. The amplified DNA of the eight plant samples were sequenced as described in Section 3.

The DNA sequences of the six *Panax* species and *M. jalapa* and *Ph. acinosa* are aligned in (Fig. 2). As expected, the sequences of the *Panax* species show a very high degree of homology in the 5.8S gene, in the range of 98.8 to 99.4% (Fig. 2). The DNA sequences of *P. japonicus* and *P. trifolius* are identical in the region. The 5.8S sequences of the two adulterants are less homologous to that of *Panax* species, but are still in the range of 96–97% (Fig. 2).

For the *Panax* species the DNA sequence in the two ITS regions are more variable than that in 5.8S region, in the range of 92.8 to 99.4%. The great majority of the variation is transition or transversion mutations, and only four cases of deletion of single nucleotide were found (Fig. 2). The variation among the *Panax* species and the two adulterants are even more evident, with less than 62% homology was detected. The inter-specific DNA variation can be used to trace the phylogenetic relationship among the *Panax* species. Our data are in agreement with the conclusion of Wen and Zimmer (1996) that *P. trifolius* is clearly separated from rest *Panax* species and *P. quinquefolius* is most closely related to *P. ginseng* as previously believed based upon the morphological similarity (Hara, 1970; Hu, 1980).

The existence of the sequence polymorphism in the ITS1 and ITS2 regions allows various restriction enzymes to create RFLP profiles characteristic to each of the six *Panax* species (Fig. 2). The RFLP patterns for the two adulterants, deduced from their sequences, were also distinct from these of the *Panax* species (Fig. 2). Three rDNA RFLP profiles, generated by *Hin*f1, *Sau*3A and *Taq*I, of *P. quinquefolius* and *P. ginseng* as well as those of the two adulterants were further studied.

DNA was isolated from 0.1 g dried roots of each of the plant samples. The rDNA covering the ITS1–5.8S–ITS2 region was amplified from total genomic DNA of *P. quinquefolius*, *P. ginseng, M. jalapa*, and *Ph. acinosa* using primers 18d and 28cc (Fig. 1), digested with one of the three restriction enzymes, and fractionated on agarose gel. It can be seen from the RFLP patterns of *Hin*fI (Figure 3A), *Taq*I (Figure 3B) and *Sau*3A (Figure 3C) that the RFLP profiles of the two ginsengs are different from each other and from these of the two adulterants. In *Hin*fI digestion (Figure 3A), the two fragments of 60 and 100 bp present in *P. quinquefolius* (lane 1) are absent from *P. ginseng* (lane 2), while the fragment of 170 bp present in *P. ginseng* is absent from *P. quinquefolius*. These three fragments are absent from the two adulterants (lanes 3 and 4). *M. jalapa* contains two characteristic fragments of 250 bp and 350 bp in size (lane 3). In *Taq*I digestion (Figure 3B), fragments of 106, 170 and 260 bp are present in *P. quinquefolius* (lane 1) but absent from *P. ginseng* (lane 2); while fragments of 230 and 280 bp are present in *P. ginseng* but absent from *P. quinquefolius*. Both adulterants *M. jalapa* and *Ph. acinosa* contain characteristic fragments of 370 and 380 bp in size, respectively (lanes 3 and 4). In *Sau*3A1 digestion (Figure 3C), in contrary to *P. quinquefolius* (lane 1), *P. ginseng* (lane 2) contains two unique fragments at the size of 120 bp and 580 bp. On the other hand, in comparison of the two *Panax* species, *M. jalapa* (lane 3) contains two fragments of 120 and 630 bp in size and *Ph. acinosa*

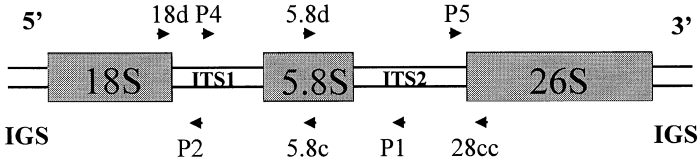

Fig. 1. The organization of plant ribosomal RNA genes (Hillis et al., 1991). The three subunits, 18S, 5.8S and 26S, are separated by internal transcribed spacers (ITS1 and ITS2). The repeat unit is separated by intergenic spacer (IGS). The sizes of ITS1 and ITS2 in *Panax* species are about 242 and 224 bp, respectively. The primers used for DNA amplification are: 18d: 5′ CAC ACC GCC CGT CGC TCC TAC CGA 3′; 5.8c: 5′ TTG CGT TCA AAG ACT CGA TG 3′; 5.8d: 5′ AAC CAT CGA GTC TTT GAA CGC A 3′ and 28cc: 5′ ACT CGC CGT TAC TAG GGG AA 3′. The primers used for DNA sequencing are P1: 5′ ATT ATC CGC CCC TCC GCC T 3′; P2: 5′ AAG GGT GGT CCC CGA CCA T 3′; P4: 5′ ATG GTC GGG GAC CAC CCT T 3′ and P5: 5′ ACC GCG CGG TTG GCC CAA AT 3′.

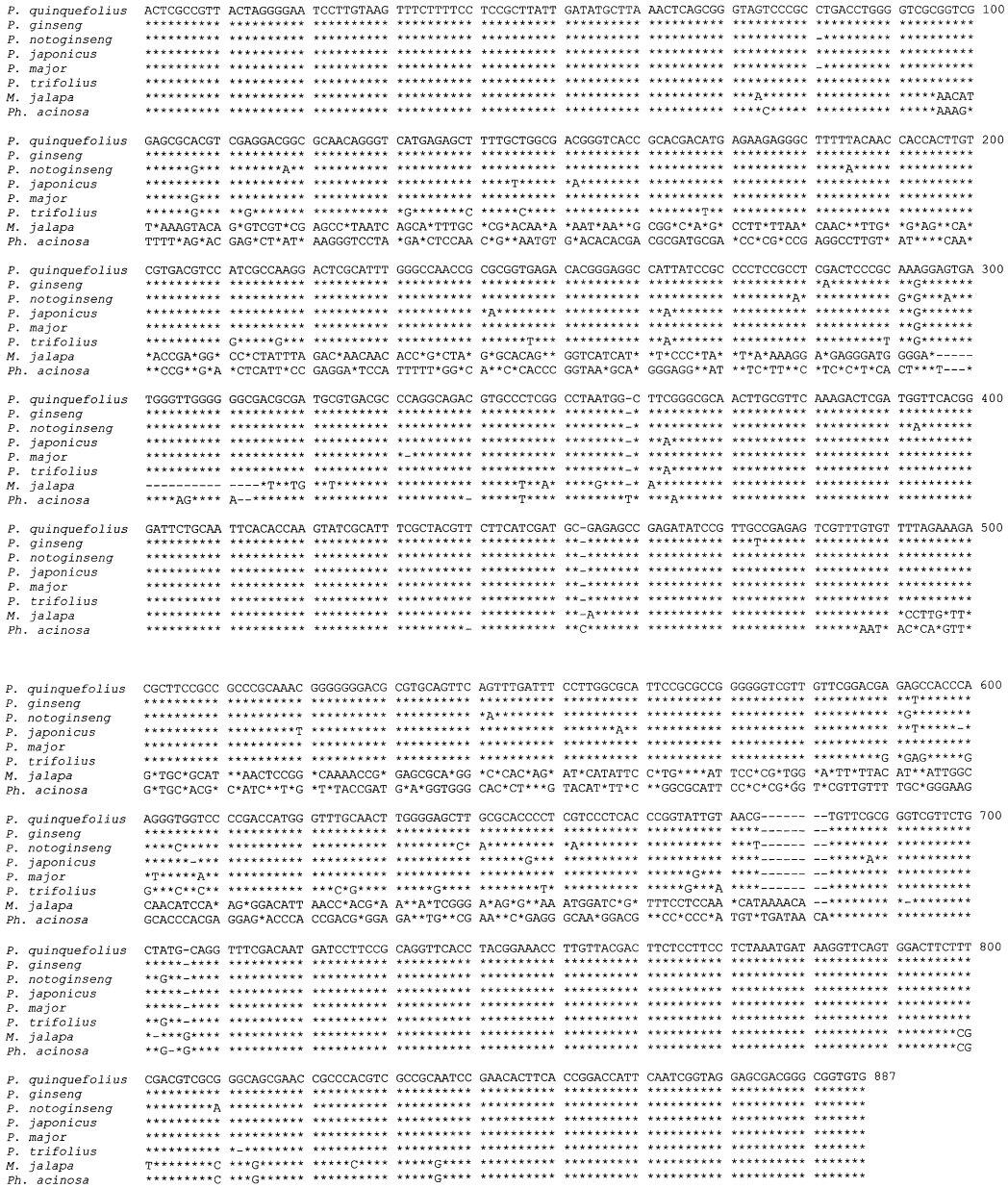

Fig. 2. The DNA sequences of *P. quinquefolius*, *P. ginseng*, *P. notoginseng*, *P. japonicus*, *P. major*, *P. trifolius*, *M. jalapa* and *Ph. acinosa* in the ITS1–5.8S–ITS2 region. Position 1 is the 5′ end of primer 28cc. For *Panax* species the 5.8S corresponds to position 247–486 bp; ITS1 487–739 bp and ITS2 100–246 bp. For the two adulterants, the 5.8S of *M. jalapa* corresponds to position 341–486 bp and 5.8S of *Ph. acinosa* 311–486 bp; ITS1 of both correspond 487–738 bp and ITS2 of *M. jalapa* 98–340 bp and ITS2 of *Ph. acinosa* 98–310 bp.

(lane 4) contains one unique fragment of 690 bp. These experimental data agreed with the RFLP patterns deduced from the DNA sequences.

In order to assess the sensitivity of the method in terms of detection of contamination, the RFLP profiles were also generated from mixed samples of *P. quinquefolius* and *P. ginseng* in different proportions. The presence of mixed RFLP patterns of *P. quinquefolius* and *P. ginseng* indicates that the impurity of the samples (Figure 3A, B and C, lanes 5 to 9). As little as 10% of contamination of *P. ginseng* can be readily detected from the *P. quinquefolius* sample (Figure 3A, B and C, lanes 5).

In plant nuclear genome, ribosomal RNA (rDNA) genes are normally clustered in an array of multiple tandemly repeated cistron of 18S–ITS1–5.8S–ITS2–26S (Wen & Zimmer, 1996). The sequence separating the 18S and 5.8S rRNA genes was designated as ITS1 (internal transcribed spacer 1) and the sequence between 5.8S and 26S was designated as ITS2. The coding regions of the three rDNA genes are highly conserved, whereas the

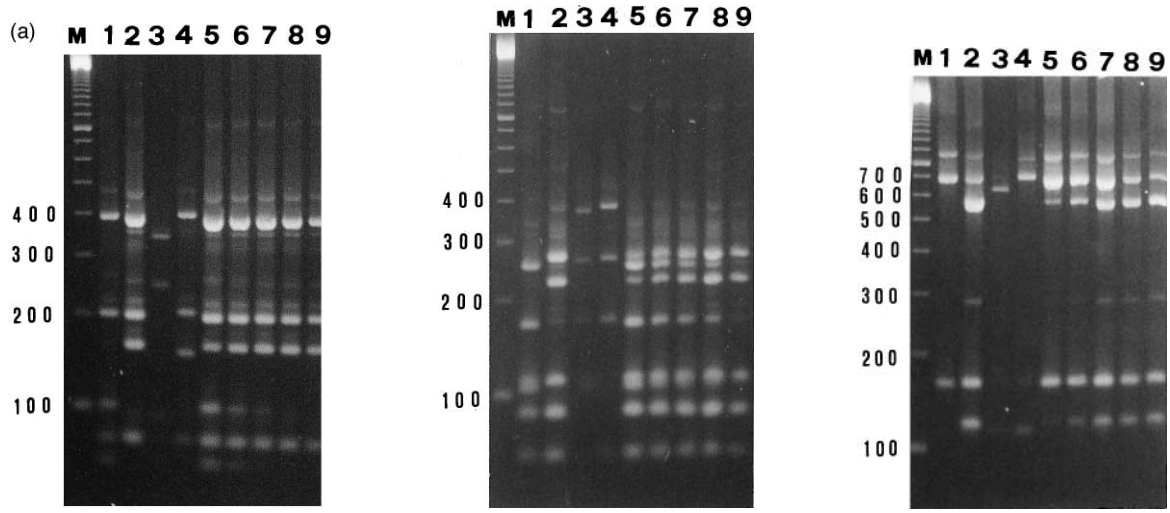

Fig. 3. The RFLP patterns of the ITS1–5.8S–ITS2 region for *P. quinquefolius*, *P. ginseng, M. jalapa* and *Ph. acinosa*. Lane M, DNA sizes marker; lane 1, *P. quinquefolius*; lane 2, *P. ginseng*; lane 3, *M. jalapa*; lane 4, *Ph. acinosa*. Lanes 5 to 9 represent the RFLP patterns of the mixed samples of herb powders from *P. quinquefolius* and *P. ginseng* in different ratio. Lane 5, *P. quinquefolius* and *P. ginseng* in the ratio of 9:1; lane 6, in the ratio of 7:3; lane 7, in the ratio of 1:1; lane 8, in the ratio of 3:7 and lane 9, in the ratio of 1:9. The RFLP was generated as described in Section 3. 3A: *Hin*fI digestion; 3B: *Taq*I digestion; and 3C: *Sau*3A1 digestion. Molecular sizes are in bp.

sequence homology within the ITS1 and ITS2 regions are lower across the plant kingdom (Yokota, Kawata, Iida, Kato, & Tanifuji, 1989). Furthermore within a given individual organism or species, the rDNA sequence is usually very similar due to the homogenisation of the sequence by gene conversion and crossing over (Arnheim et al., 1980; Zimmer, Martin, Beverley, Kan, & Wilson, 1980; Appels & Dvorak, 1982; Arnheim, 1983; Hillis, Moritz, Porter, & Baker, 1991; Baldwin et al., 1995). The sequence data of *Panax* species reported here agree well with these notion.

Our sequence data of *P. quinquefolius* and *P. ginseng* are identical to the recently published sequences (Wen & Zimmer, 1996). This indicates that ITS1–5.8S–ITS2 in these two species are consistent among different individual samples and thus makes the measurement of RFLP in this region an accurate and reliable approach to differentiate the two plants. In this communication we have demonstrated that this method is indeed capable of authentication of *P. quinquefolius* and *P. ginseng*, and differentiating them from each other and from two of their common adulterants Fig. 3. We have sequenced the ITS1–5.8S–ITS2 region of two additional *P. ginseng* samples collected from different locations and a variation of one nucleotide was detected in one of the samples (unpublished results). Our recent data (unpublished results) and the data reported by others (Bai, Brandle, & Recleder, 1997) indicate that genetic variation exists among the different cultivars or individual samples of *P. quinquefolius*. These variations may or may not locate in the ITS1-5.8S-ITS2 region and effect the RFLP used for the authentication; nevertheless, it is prudent to use RFLP of more than one restriction enzyme in authentication of plant samples to avoid mis-diagnosis.

In comparison to the traditional and other existing methods of authentication of *Panax* species, the procedure reported here offers the following advantages: (1) the authentication results are reliable and reproducible, and are not affected by the physical forms and physiological conditions of the plant samples; (2) it is a method of high sensitivity: micrograms of the herb sample is sufficient; (3) using different enzymatic digestion, more than one distinctive RFLP profiles can be generated thus provide additional markers of identification and (4) the contamination of non-authentic materials can be detected. We are currently expanding the application of the molecular authentication method into many other traditional Chinese medicinal herbs. Similar to the situation of *Panax*, very limited if any DNA markers are available from these plants. We find that ITS1 and ITS2 regions can be readily amplified from these plant samples using the flanking primers described in this article (unpublished results).

3. Experimental

3.1. Plant materials

Dried roots of *P. ginseng*, *P. notoginseng* and *P. major* were from China; *P. japonicus* was from Japan; *P. quinquefolius* and *P. trifolius* were from the USA and *M. jalapa* and *Ph. acinosa* were from Hong Kong.

3.2. DNA extraction and rDNA amplification

The plant DNA was extracted essentially as described (Draper & Scott, 1988). In the mixed sampling assay,

powders of *P. quinquefolius* and *P. ginseng* were mixed in different proportions of 9:1, 7:3, 1:1, 3:7 and 1:9 prior to the extraction. The plant rDNA was amplified using a pair of primers 18d and 28cc (Hillis & Dixon, 1991), which correspond to the conserved regions of plant 18S and 26S, respectively Fig. 1. The PCR reaction was performed in a 50 μl mixture containing 1 ng plant DNA, 1 × *Taq* buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 0.2 mM dNTPs, 1 μM of each primer and 1 unit of *Taq* polymerase. Initial template denaturation was programmed at 94°C for 5 min. It was then subjected to 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and with a final extension of 72°C for 10 min.

3.3. rDNA sequencing

The rDNA of the six *Panax* species were sequenced twice using two methods. The rDNA of the *Panax* species and the two adulterants were initially subcloned into TA vector (Invitrogen) and sequenced using T7 sequencing kit (Pharmacia) or USB Sequenase kit (Amersham). The sequences of the six *Panax* samples were subsequently verified by direct PCR sequencing of both strands of the DNA using SequiTherm Cycle sequencing kit (Epicentre Technologies). The locations and sequences of different primers used in the sequencing are shown in Fig. 1. The sequences were aligned manually and the percentage identity was calculated by the computer program DNASIS (version 7, Hitachi).

3.4. Generation of RFLP profiles

Plant rDNA amplified using primers 18d and 28cc were purified using Geneclean kit (Bio101) and digested with restriction endonucleases *Taq*I, *Sau*3AI or *Hin*fI. 1.5 μg rDNA was used for each digestion in a volume of 50 μl. The digestion products were purified by phenol:chloroform:isoamyl alcohol (25:24:1) and resolved in a 3% agarose gel.

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